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UNIVERSITY OF ALBERTA

DNA FLOW CYTOMETRY:
TECHNICAL IMPROVEMENTS AND APPLICATIONS
IN TUMORS OF BREAST AND CERVIX

by

JEANNETTE CHRISTINE BABIAK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND

RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

DEPARTMENT OF PATHOLOGY

EDMONTON, ALBERTA

FALL, 1991

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DEGREE: **Master of Science**

YEAR THIS DEGREE GRANTED: **Fall 1991**

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The undersigned certify they have read and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **DNA FLOW CYTOMETRY: TECHNICAL IMPROVEMENTS AND APPLICATIONS IN TUMORS OF BREAST AND CERVIX** submitted by **Jeannette C. Babiak** in partial fulfillment of the requirements for the degree of **Master of Science** in **Experimental Pathology**.

Dr. Sibrand Poppema

Dr. Locksley McGann

Dr. Joan Allalunis-Turner

DATE: September 15, 1991

DEDICATION

To My Family...
especially my Mother.

Without their love, patience
and concern,
this accomplishment would
never have been
achieved.

ABSTRACT

Flow cytometry has rapidly emerged as a powerful new method for quantitating cellular DNA content. A significant advance in DNA flow cytometry was the development of a technique for analysis of paraffin-embedded archival material permitting retrospective clinical studies of several different tumor types. Nevertheless, there is considerable variation among laboratories in techniques of cell preparation, staining and data interpretation. This thesis has addressed the variability inherent in the paraffin procedure and provided technical improvements, most notable being the development of an automated deparaffinization and rehydration procedure.

Using this improved technology, this thesis also explored the prognostic value of cellular DNA content in two specific tumor groups. In axillary node-negative breast cancer patients, DNA measurements were evaluated for their ability to identify individuals at increased risk of recurrence. Flow cytometric DNA analysis was also used to correlate the inherent radiosensitivity of cervical and endometrial carcinomas with the DNA index. In these specific groups, DNA ploidy was not found to have significant predictive value of tumor behavior. This suggests that the biological behavior of tumors does not have a simple relationship to DNA ploidy, and that well-established prognostic variables should be examined simultaneously with DNA content to help develop a more quantitative understanding of tumor cell heterogeneity.

ACKNOWLEDGEMENT

I would like to recognize and express my sincerest gratitude to the following individuals whose contributions to this thesis were very much appreciated.

To Dr. Sibrand Poppema, my supervisor and mentor, for his continued support and guidance. His infinite wisdom and creative thinking inspired me immeasurably.

To Dr. Locksley McGann, for his friendship and service on my committee. His unfailing words of encouragement and pursuit of excellence guided my learning to the fullest.

To Dr. Joan Allalunis-Turner, my committee member, for accelerating my interest research in the scientific community.

To Dr. Mary-Beth Yachyshyn and Dr. Judith Hugh for their preceptorship along the way.

To Dr. Robert Stinson, Mr. John Marken, Susan Todd, Barry Hunt and Judy Tween for their departmental assistance.

To Richard Besse, Frank LoCicero and Brian Brady for their excellence in audio-visual services.

To Jan Spivak-Steele for her technical assistance, but above all, her friendship and understanding.

To Dr. Ron Moore for travelling a similar path and being a role model to follow.

To Cindy Johns, who I am especially grateful to, for typing and coordinating this manuscript. Her resourcefulness, patience, and genuine devotion were responsible for the scheduled completion of this thesis.

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ABBREVIATIONS

CV	-	coefficient of variation
DANS	-	1-Dimethylamino-naphthalin-S-sulfochloride
DI	-	DNA index
DMEM	-	Dulbecco's modified eagle's media
DNA	-	deoxyribonucleic acid
EGFR	-	epidermal growth factor receptor
ER	-	estrogen receptor
FIGO	-	International Federation of Gynecologists and Obstetricians
FITC	-	fluorescein isothiocyanate
FNA	-	fine-needle aspiration
HBSS	-	Hank's Balanced Salt Solution
PBS	-	phosphate buffered saline
PEG	-	polyethylene glycol
PI	-	propidium iodide
RNA	-	ribonucleic acid
SEM	-	standard error of the mean
UV	-	ultraviolet

CHAPTER ONE

INTRODUCTION

1.1 OBJECTIVES

The ability to detect and measure several cellular constituents quickly and accurately has made flow cytometry a very valuable tool with numerous potential applications. In particular, the measurement of the relative DNA content of cells has assisted pathologists in the detection and evaluation of malignant disease. It offers pathologists a powerful new method which can be applied especially for diagnosis, prediction of prognosis and measurement of response to therapy of tumors. For several different types of malignancies including breast[1-3], cervix[4,5], colon[6-9] and ovary[10-12], the prognostic significance of DNA ploidy has been investigated. The presence of DNA aneuploidy in these primary tumors predicts for tumor recurrence and shortened disease-free survival suggesting that aneuploidy is often a sign of poor prognosis. Measurement of cellular DNA content is also used to quantitate the percentage of cells in the DNA-synthetic phase, thus reflecting the proliferative activity of the tumor. Development of a technique for the analysis of paraffin-embedded archival material was a milestone in DNA flow cytometry allowing retrospective studies to determine the prognostic value of cellular DNA[13]. The technique, however, has significant limitations due to the variability among laboratories in assay procedures, staining protocols and data interpretation. The objective of this thesis, is to address some of the technical difficulties of processing paraffin-embedded tissue for flow cytometric DNA analysis, and to introduce improvements to minimize the variation inherent in this technique (Chapter 3).

Using this improved methodology, the application of DNA flow cytometry in solid tumors of breast and cervix is also explored in this thesis. The study of breast tumors (Chapter 4) was driven by the important clinical need for better prognostic

indicators and for accurate predictors of relapse in node-negative breast cancer patients. The unpredictable natural history of node-negative breast cancer has resulted in significant heterogeneity in the clinical outcome of these patients despite demonstrating morphologically and biochemically similar tumors. An overall relationship between DNA aneuploidy and an unfavorable prognosis has been reported in primary breast neoplasms. Given that the majority (60-70%) of primary breast tumors are aneuploid[2,14-18], DNA ploidy analysis, with c-*erb*B-2 oncogene analysis, was investigated for its clinical utility as a second-generation prognostic parameter to help identify a subgroup of node-negative breast cancer patients at increased risk of tumor recurrence.

The analysis of cellular DNA content may also have important therapeutic implications in patients with gynecological malignancies. When carcinoma of the cervix is confined to the pelvis, it is potentially curable by radiotherapy. Flow cytofluorometric studies have suggested that aneuploid cervical tumors are more radioresponsive than are diploid tumors conferring a significantly better prognosis in patients with abnormal DNA stemlines[19]. In Chapter 5, the possibility that tumor DNA ploidy may be correlated with the inherent radiosensitivity of cervical carcinoma is explored.

1.2 DEFINITION OF FLOW CYTOMETRY

Cytometry refers to the measurement of physical and/or chemical characteristics of individual cells and cellular constituents. The operative word is individual. Each cell or cellular organelle is considered as a distinct, discrete entity that is measured separately. Cytometry concentrates on the single cell as the measuring unit, but allows the simultaneous measurement of several cellular parameters. These measurements may range from simple properties (such as size,

internal complexity, viability) through measurements of biochemical constitution (such as DNA or RNA content, expression of surface antigens) to complex measures of physiologic status and enzyme activity. Flow cytometry, as the term implies, is a process in which such measurements are made while the cells pass single file through the measuring apparatus in a fluid stream.

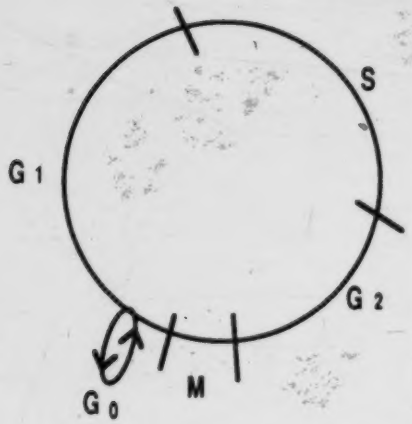
1.3 PRINCIPLES OF DNA FLOW CYTOMETRY

The measurement of cellular DNA content by flow cytometric analysis is currently the application of greatest pathological interest. Two principal measurements are derived from flow cytometric DNA analysis: (1) cell cycle analysis, and (2) ploidy analysis. The ability to measure DNA content by flow cytometry is based on three principles: (1) cells in different stages of the cell cycle have different but predictable amounts of DNA; (2) fluorochromes are available that bind stoichiometrically to DNA and (3) normal cells are diploid having a known amount of DNA, whereas malignant cells tend to be aneuploid.

1.3.1 Concept of the Cell Cycle

The classical view of the mammalian cell cycle was deduced through autoradiographic studies of the incorporation of DNA precursors[20] (Figure 1.1). Once initiation has occurred, cells enter a DNA-synthetic phase (S-phase) during which the complete complement of chromosomes is duplicated. Cells produce increasing amounts of DNA as they transit this phase. Upon completion of DNA synthesis, a phase of apparent inactivity in DNA synthesis, or a gap phase (G_2), is entered. At this point in the cycle, cells have two sets of chromosomes, and consequently, twice the normal amount of DNA (4N). The G_2 phase terminates with mitotic division (M) resulting in two daughter cells which also enter a gap

The Cell Cycle



G_1 PHASE	-	RNA + PROTEIN SYNTHESIS 2N
S PHASE	-	DNA SYNTHESIS 2N → 4N
G_2 PHASE	-	RNA + PROTEIN SYNTHESIS PRIOR TO CELL DIVISION (4N)
M PHASE	-	MITOSIS (CELL DIVISION) DAUGHTER CELLS PRODUCED (2N)
G_0 PHASE	-	RESTING STAGE (2N)

FIGURE 1.1

Schematic representation of the mammalian cell cycle. By definition, the cell cycle is the interval between two consecutive mitoses in a continually dividing cell.

phase, G_1, and have a normal chromosomal complement (2N). Cells commonly revert back into a quiescent or non-proliferating state designated G_0 or G-null. Like those in the G_1 phase, these resting cells have diploid DNA content. The data derived from flow cytometric DNA analysis are often compiled into frequency distribution histograms (Figure 1.2). These histograms demonstrate the discrete cell cycle compartments that correspond to the various cell cycle phases. The measurement of cells in each of these compartments allows the rapid calculation of the relative proportion of cells in the presynthetic (G_0/G_1), synthetic (S) and post-synthetic (G_2M) phases of the cell cycle.

1.3.2 DNA Fluorochromes

The second principle enabling the measurement of DNA content by flow cytometric analysis is the availability of specific fluorochromes that bind to DNA in a stoichiometric fashion. Among the available dyes (ethidium bromide, propidium iodide, DAPI), propidium iodide is the most widely used in flow cytometric analysis. Its intercalative binding to double-stranded nucleic acids results in an approximate 100-fold increase in fluorescence, the emission of which is proportional to the amount of DNA present in a cell[21].

1.3.3 Quantitation of Cellular DNA Content

Third, our ability to quantify cellular DNA is based on early static microphotometric observations[22-24]. Such measurements have shown that normal human cells have a diploid amount of DNA characterized by a fixed set of chromosomes, whereas abnormal (or malignant) cells tend to have an aneuploid content of DNA. Most often, the aneuploidy of malignancy, a result of the addition

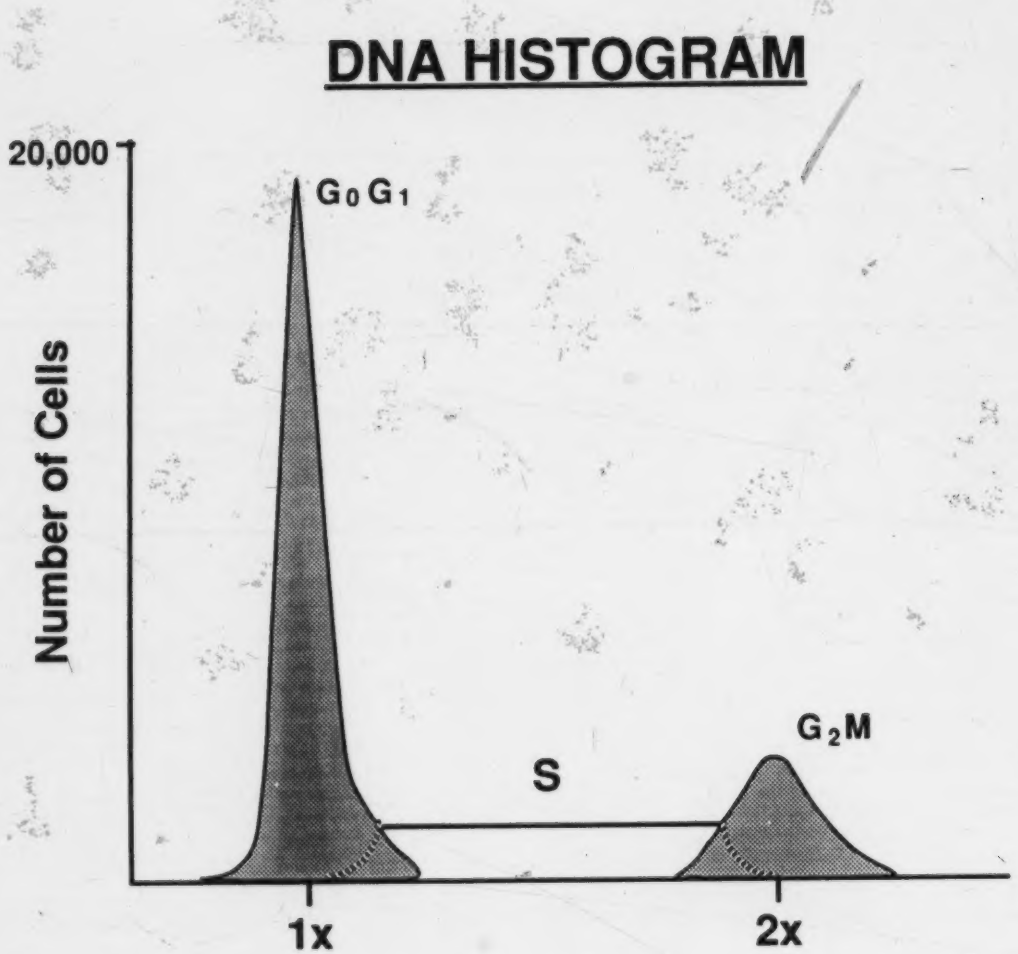


FIGURE 1.2

Representative DNA histogram corresponding to the various cell cycle compartments. The number of particles is given on the vertical axis versus the distribution of fluorescence intensity within a population along the x-axis.

or loss of chromosomes or the creation of new chromosomes from balanced or unbalanced translocations, demonstrates measurable changes in the cellular DNA. This change in cellular DNA content is expressed by the DNA index (DI). This term refers to the ratio of the DNA content of the G_0/G_1 cells in the population being studied to the G_0/G_1 cells of a reference sample, when normal human diploid cells or nuclei are the reference. It is calculated by dividing the mean or modal channel number of the G_0/G_1 peak in the DNA histograms of the test population by the mean or modal channel number of the G_0/G_1 peak of the diploid reference cells. By definition, the DNA index of normal diploid cells is 1.00. Aneuploid tumors containing less than the diploid content are hypodiploid ($DI < 1.00$) or, more commonly, have more than the normal amount of DNA and are referred to as hyperdiploid ($DI > 1.00$). The term "tetraploid" has been reserved for populations demonstrating twice the normal diploid DNA content ($DI = 2.00$). At present, the resolution of flow cytometers is such that two clonal cell populations can be resolved providing there is at least four percent difference in their DNA content[25].

1.4 PRODUCING MONODISPERSE SUSPENSIONS FOR FLOW CYTOMETRIC DNA ANALYSIS

The quality of DNA histograms from flow cytometric analysis is often dependent on the technique used to produce the monodisperse suspension of cells or nuclei. The endpoint to be measured is the major determinant of which dissociation technique should be used, not convenience, not past experience, nor availability of reagents. Optimally, the technique chosen should meet the following criteria: (1) no preferential loss of subpopulations of tumor or normal cells; i.e., the *in-vitro* sample is representative of the *in-situ* sample; (2) minimal debris and clumping of cells or nuclei; (3) optimal cell yields per gram of tissue and (4) retention of desired morphologic, molecular and biochemical phenotypes under

study. The ability to meet these objectives varies with the source of tissue and the means of dissociation. Hence, the dissociation technique chosen must be individualized for the tissue and cellular parameters under study.

1.4.1 Fine Needle Aspiration

Fine needle aspiration (FNA) is one method of obtaining cellular material from solid tumors for DNA flow cytometry. Since FNA results in a gentle mechanical dissociation, single cells rather than segments of tissue are obtained. The amount of diagnostic material in FNA samples varies with the size, location, nature of the lesion, the number of passes made, and the experience and skill of the person performing the aspiration. FNA and DNA flow cytometry have been used together in various clinical settings, particularly for sampling of breast lesions for diagnosis. The detection of DNA aneuploid stemlines and the quality of DNA histograms from FNA samples are significantly better than results obtained from mechanical or enzymatic dissociation of the same solid tumors. Another advantage of FNA, other than yielding a monodisperse suspension of cells, is the ability to inject the needle's contents directly into the staining solution thereby eliminating extensive processing for DNA flow cytometry.

1.4.2 Solid Tumor Dissociation

The dissociation of solid tissue is a major obstacle in the routine clinical application of flow cytometric DNA analysis of solid tumors. Formation of a single cell suspension from a solid tumor requires an alteration or destruction of the elements involved with tissue and cellular cohesion. Current methods use chemical, mechanical, or enzymatic techniques alone, or in combination, to derive intact, viable cells from a solid tumor. Although a large number of specimens from widely

divergent tissue types are commonly processed by one preparative method, to date no standard recommendations for dissociating solid tumors have been established. Comparative studies of the available procedures are needed to document the most optimal method of obtaining a representative cellular suspension from a specific tumor type. Once this standardization is achieved, flow cytometric studies of solid tumors will reflect the properties of the tumor cells *in-vivo* and enable investigators to accurately compare results from different laboratories.

1.4.3 Paraffin-Embedded Technique

One of the most significant developments in clinically oriented flow cytometry was the demonstration that meaningful flow cytometric analysis of DNA could be performed on nuclear suspensions recovered from thick sections of formalin-fixed, paraffin-embedded tissue blocks. This method of processing archival material, developed in 1983 by Hedley *et al.*[13], is used as a means to overcome several of the disadvantages of using fresh tissue for flow cytometric analysis. These include: (1) flow cytometric analysis of fresh material is precluded when the sample has been submitted in total for histopathology, or when the need for flow cytometry at the time of sampling is not realized; (2) the possibility of increased sampling error by the inability to select specific histopathologic regions using fresh tissue and (3) the inability to perform retrospective studies with fresh tissue samples to determine the prognostic significance of DNA content.

1.5 LIMITATIONS OF THE PARAFFIN PROCEDURE

The processing of paraffin material for flow cytometric DNA analysis has some limitations. These limitations were individually addressed in this thesis. First, DNA histograms from archival material, in general, are of poorer quality than those

obtained from a fresh specimen of the same sample. This reduced quality arises from increased coefficients of variation (CV) and technical factors such as: type and duration of tissue fixation, tissue cellularity, section thickness and enzymatic digestion. These factors impact on the amount of debris present. Increased debris often precludes accurate analysis of S-phase and G_2M fractions and may obscure the presence of diploid populations. Diploid cells that are low in number may be hidden by debris, leading to the interpretation of a DNA aneuploid cell population as being DNA diploid. Overall, high CVs and increased debris give rise to variability and discordance in several ways: (1) in detecting DNA aneuploid cell populations and cell proliferation; (2) in interpreting DNA histograms and (3) in determining the acceptability of a histogram. Next, the use of paraffin-embedded material precludes the use of a reliable standard for the determination of DNA ploidy. Alone this is the single most significant disadvantage of the paraffin technique. Third, the labor inherent in the sample preparation makes the paraffin procedure tedious and time-consuming. The fourth disadvantage of using archival material for flow cytometric DNA analysis is the lack of standardized assay procedures to process this tissue. Having a variety of techniques not only contributes to result variability but hampers attempts to compare results from different laboratories. Last, the uncertainty in the definition of DNA aneuploidy has resulted in a lack of consensus on DNA histogram classification[26].

1.5.1 Increased Coefficients of Variation

The precision and quality of flow cytometric measurements are commonly expressed by the coefficient of variation (CV), defined as the standard deviation of the peak divided by the mean channel number of the peak. The CV measures the distribution of fluorescence or light scattering intensities from "nearly identical" particles. The particles may be biological, i.e., fixed stained cell nuclei or artificial,

i.e., fluorescent polystyrene microspheres. For most flow cytometric measurements, CVs below 5% are acceptable; however, for DNA measurements of fresh tissue, CVs below 3% are desired since DNA content is so precisely regulated as to vary less than 2% from cell to cell in homogeneous, non-dividing populations[27]. Unfortunately, the use of formalin-fixed paraffin-embedded material often yields wide fluorescence distributions attributed to cell-to-cell differences in dye uptake, and therefore increased CVs. This is due primarily to the crosslinking between DNA and nucleoproteins during tissue fixation. Formalin causes the formation of methylene-bridges between two neighboring amino groups resulting in complexes in which DNA-protein interaction is no longer in its native ionic state, but rather in a covalent one[28]. These covalent linkages affect the accessibility and uniform binding of DNA to the dye. Thus, DNA histograms that demonstrate "wide CV diploid" peaks may well be composites of diploid and unrecognized near-diploid populations raising the possibility that aneuploid stemlines may be overlooked with the use of archival material.

1.5.2 Tissue Fixation

Tissue fixation is one important cause of the many problems associated with the use of archival material for DNA flow cytometry. Since fixation lags behind the rate of penetration, large tissue samples may undergo autolysis in central regions. During tissue autolysis, nuclear proteins degrade causing enhanced binding of fluorescent dyes to DNA. This increased intensity of staining can be a source of false aneuploid peaks in DNA content analysis[29]. Also, shorter periods of fixation may lead to so called "schizophrenic" fixation with central portions of the tissue that are only fixed in alcohol during the dehydration procedure. For these reasons, a bimodal DNA histogram may not always indicate the presence of an abnormal nuclear DNA content but instead may represent variable staining patterns. A

second factor to consider when preparing tissue for DNA flow cytometric analysis is the choice of fixative, particularly since mercury-based ones lead to DNA histograms with increased CVs and high baseline debris.

1.5.3 Tissue Cellularity

The ability to insure adequate cellularity from specific histopathologic regions of a lesion is a major advantage of using archival material; however, tissue cellularity is often a forgotten variable when preparing samples for flow cytometric DNA analysis. The quality of results is increased by careful sampling of the paraffin blocks. Adequate sampling ensures that the relative proportion of normal and tumor cells is sufficient for analysis and second, that the amount of debris and necrotic tissue is minimized.

1.5.4 Tissue Sectioning

Sectioning of the paraffin block is the single most important procedural step affecting the final quality of DNA histograms from archival material. Sectioning results in fragments of cut nuclei; the amount being is inversely related to the section thickness and nuclear size. With thin sections, there is a progressive increase in baseline nuclear debris and a progressive decrease in the relative number of aneuploid cells. The former is explained by the increased probability of transecting nuclei with thinner sections, and the latter by the greater likelihood of transecting larger (aneuploid) nuclei. Both artifacts can be minimized by using an optimal section thickness.

1.5.5 Enzymatic Digestion

Enzymatic digestion of rehydrated sections also has a major impact on the quality of subsequent DNA histograms. This step is required to obtain a nuclear suspension and to break down the covalent crosslinks between nucleoproteins and DNA. Once disrupted, the accessibility of the DNA-binding-dye into the nucleus is markedly enhanced. On the other hand, the enzymatic digestion may degrade the nuclear material resulting in increased amount of debris if samples are subject to prolonged incubation.

1.5.6 Lack of Reliable Standards

In the analysis of fresh and frozen samples for DNA analysis, a variety of different cell types have been used as standards. Use of a standard involves comparing the DNA-related fluorescence of a test population to that of a standard with known DNA content. Human lymphocytes are commonly used because of their diploid DNA content and tight G_0/G_1 fluorescent distributions. However, in cases where abnormal cells with very near diploid DNA content are admixed with a standard of normal DNA content, the result may be a broadening of the G_0/G_1 peak without the appearance of discrete normal and abnormal peaks. In such cases, use of standards with lower than the normal diploid content are used. These include chicken or trout red blood cells which have a respective fluorescence of 0.35 and 0.80 of that emitted by diploid mammalian cells[30].

In paraffin samples, the effects of tissue fixation and enzymatic digestion on DNA staining vary to such a degree they preclude the use of reliable standards. Formalin fixation decreases and enzyme digestion increases the intensity of DNA staining, and the extent to which this occurs varies between cell types. This variability in staining intensity results in an inconsistent ratio of fluorescence of the

diploid cells in the paraffin block to that of an external standard. It is therefore recommended that the G_0/G_1 peak with the lowest DNA content is assumed to be diploid when determining DNA index from paraffin blocks. This recommendation originates from reviewing flow cytometric studies of fresh material and finding an overwhelming majority of abnormal stemlines to be hyperdiploid[31]. In practice, this assumption works with the limitation that hypodiploid tumors cannot be identified with confidence using paraffin-embedded material.

1.5.7 Decreased Efficiency

In addition to the reduced quality of DNA histograms and lack of reliable standards, another major drawback encountered with paraffin-embedded material for flow cytometric DNA analysis is the time and labor involved in sample preparation. The deparaffinization and rehydration of tissue sections is the most time-consuming step of the procedure. Sections must be dewaxed in three changes of xylene and rehydrated in a series of graded ethanol solutions. The need for repeated centrifugations and aspirations makes this step very labor-intensive. Second, the manual preparation of reagents is lengthy and involves exposure to potentially harmful aerosols. A consistent concentration of propidium iodide, a suspect mutagen, must be maintained with each new batch of staining solution to ensure reproducible binding of PI to DNA, and to provide a fluorescence ratio that consistently matches DNA content. Moreover, since PI binds by intercalation to double-stranded nucleic acids[32], it is necessary to remove the interfering RNA with an enzyme such as RNAse. However, prior to the use of RNAse, pretreatment of the enzyme at 90^{o}C for 30 minutes is required. This is necessary to remove residual DNAse activity present in the RNAse that may be responsible for DNA degradation.

1.5.8 Lack of Standardized Assay Procedures

The lack of standardized assay procedures has also contributed to widespread variability in DNA flow cytometry. Most of laboratories have developed their own methods for sample preparation or devised modifications of the more common procedures. While laboratories may have achieved intralaboratory precision by perfecting and quality controlling their methods, the problem of technique and result inconsistency between laboratories remains. At present, numerous diluents, ranging from simple to complex saline solutions, have been used with PI despite evidence suggesting that the stoichiometric staining property of PI is affected by salt concentration[32]. Second, depending on the laboratory, the digestion of RNA is often carried out for varying time periods (30 to 60 minutes) and at variable temperatures (24°C or 37°C). Futhermore, the quality of RNAse used varies with respect to the units of enzyme activity and the amount of contaminating proteolytic enzymes present. Standardization is the key to accurate DNA analysis and a single, reproducible method for preparing samples will significantly reduce the variability that persists in DNA flow cytometry.

1.5.9 Variability in Data Interpretation

Yet another prevailing inconsistency in flow cytometric DNA analysis is in the definition of DNA aneuploidy. Some authors consider flow cytometric DNA histograms to be aneuploid if they contain an arbitrarily selected percentage of cells outside the diploid range, or if the diploid peak is skewed or broad, even if no discrete, definite abnormal G_0/G_1 peak is apparent[26]. This interpretation obscures the distinction between a rapidly dividing population and a clearly aneuploid one. Both patterns may indicate the presence of a tumor, but may have very different implications with respect to the biologic potential of the malignancy[33]. A

consequence of the uncertainty in defining DNA aneuploid populations is a lack of consensus on DNA histogram classification. This variability in histogram interpretation may well result in widely varying percentages of DNA aneuploidy reported in the literature even if the data are similar.

1.6 EXPERIMENTAL APPROACH

The objective of this thesis is to improve the methodology of processing paraffin-embedded tissue for flow cytometric DNA analysis. The first approach to be taken is to develop a standardized method for sample preparation to reduce the variability that is common place in the practice of DNA analysis. The next step is to interpret the cytometric data objectively using nomenclature and guidelines specific for DNA flow cytometry. These approaches will offer significant advantages over current methods for preparing and analyzing archival material for flow cytometric DNA measurements. This experiment will also explore the prognostic value of cellular DNA content in specific tumor types and provide novel evidence regarding the clinical significance of DNA measurements in solid tumors of breast and cervix and endometrium.

REFERENCES

1. Hedley DW, Rugg CA, Ng AB, Taylor IW. Influence of cellular DNA content on disease-free survival of stage II breast cancer patients. Cancer Res 44:5395-5398, 1984

2. Kallioniemi O-P, Hielanen T, Mattila J, Lehtinen M, Lauslahti K, Koivula T. Aneuploid DNA content and high S-phase fraction of tumor cells are related to poor prognosis in patients with primary breast cancer. Eur J Cancer Clin Oncol 23:277-282, 1987

3. Dressler LG, Seamer LC, Owens MA, Clark GM, McGuire ML. DNA flow cytometry and prognostic factors in 1331 frozen breast cancer specimens. Cancer 61:420-427, 1988

4. Jakobsen A. Prognostic impact of ploidy level in carcinoma of the cervix. Am J Clin Oncol 7:475-480, 1984

5. Jakobsen A. Ploidy level and short time prognosis of early cervix cancer. Radiother Oncol 1:271-275, 1984

6. Armitage NC, Robins RA, Evans DF, Turner DR, Baldwin RW, Hardcastle JD. The influence of tumor cell DNA abnormalities on survival of colorectal cancer. Br J Surg 72:828-830, 1985

7. Kohal W, Sheibani K, Terz J, Harada R. Tumor DNA content in the prognosis of colorectal carcinoma. JAMA 255:3123-3127, 1986

8. Schutte B, Reynders MM, Wiggers T, Arends JW, Volovics L, Bosman FT, Blijham GH. Retrospective analysis of the prognostic significance of DNA content and proliferative activity in large bowel carcinoma. Cancer Res 47:5494-5496, 1987

9. Wolley RC, Schreiber K, Koss LG, Karas M, Sherman A. DNA distribution in human colon carcinoma and its relationship to clinical behavior. J Natl Cancer Inst 69:15-22, 1982

10. Blumenfeld D, Braly PS, Ben-Ezra J, Klevecz RR. Tumor DNA content as a prognostic feature in advanced epithelial ovarian carcinoma. Gynecol Oncol 27:389-402, 1988

11. Friedlander ML, Hedley DW, Swanson C, Russell P. Prediction of long-term survival by flow cytometric analysis of cellular DNA content in patients with advanced ovarian cancer. J Clin Oncol 6:282-290, 1988

12. Rodenburg CJ, Cornelisse CJ, Heintz PA, Hermans J, Fleuren GH. Tumor ploidy as a major prognostic factor in advanced ovarian cancer. Cancer 59:317-323, 1987

13. Hedley DW, Friedlander ML, Taylor IW, Rugg C, Musgrove E. Method of analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 31:1333-1335, 1983

14. Kallioniemi O-P, Blanco G, Alavaikko M, Hietanen T, Mattila J, Lauslahit K, Lehtinen M, Koivula T. Improving the prognostic value of DNA flow cytometry in breast cancer by combining DNA index and S-phase fraction. Cancer 62:2183-2190, 1988

15. Muss HB, Kute TE, Case LD, Smith LR, Booher C, Long R, Kammire L, Gregory B, Brockschmidt JK. The relation of flow cytometry to clinical and biologic characteristics in women with node-negative primary breast cancer. Cancer 64:1894-1900, 1989

16. Owainati AA, Robins RA, Hinton C. Tumor aneuploidy, prognostic parameters and survival in primary breast cancer. Br J Cancer 55:449-454, 1987

17. Thorud E, Fossa SD, Vaage S, Kaalhus D, Knudsen OS, Bormer O. Primary breast cancer flow cytometric DNA pattern in relation to clinical and histopathologic characteristics. Cancer 57:808-811, 1986

18. Hedley DW, Friedlander ML, Taylor IW. Application of DNA flow cytometry to paraffin-embedded archival material for the study of aneuploidy and its clinical significance. Cytometry 6:327-333, 1985

19. Dyson JD, Joslin CF, Quirke P, Rothwell RI, Bird CC. Quantitation by flow cytofluorometry of response of tumors of the uterine cervix to radiotherapy. Br J Radiol 58:41-50, 1985

20. Howard A, Pelc SR. Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosomal breakage. Heredity Suppl 6:261-273, 1953

21. Waggoner AS. Fluorescent probes for cytometry. In Flow Cytometry and Sorting. 2nd Edition. Edited by MR Melamed, TL Lindmo, ML Mendelsohn. John Wiley & Sons, New York, pp 209-225, 1990

22. Atkin NB, Richards BM. Deoxyribonucleic acid in human tumors as measured by microspectrophotometry of Feulgen stain: A comparison of tumors arising at different sites. Brit J Cancer 10:769-786, 1956

23. Bohm N, Sandritter W. DNA in human tumors: A cytophotometric study. Curr Top Pathol 60:152-214, 1975

24. Leuchtenberger C, Leuchtenberger R, Davis A. A microspectrophotometric study of the deoxyribose nucleic acid (DNA) content in cells of normal and malignant human tissue. Am J Pathol 30:65-85, 1954

25. Vindelov LL, Christensen IJ, Jensen G, Nissen NI. Limits of detection of nuclear DNA abnormalities by flow cytometric DNA analysis. Results obtained by a set of methods for sample-storage, staining and internal standardization. Cytometry 3:332-339, 1983

26. Joensuu H, Kallioniemi O-P. Different opinions on classification of DNA histograms produced from paraffin-embedded tissue. Cytometry 10:711-717, 1989

27. Shapiro HM. Practical Flow Cytometry. 2nd Edition. Alan R. Liss, New York, 1988

28. Brutlag D, Schlehuber C, Bonner J. Properties of formaldehyde treated nucleohistone. Biochem 8:3214-3218, 1969

29. Alanen KA, Joensuu H, Klemi PJ. Autolysis is a potential source of false aneuploid peaks in flow cytometric DNA histograms. Cytometry 10:417-425, 1989

30. Vindelov LL, Christensen IJ, Nissen NI. Standardization of high-resolution flow cytometric DNA analysis by the simultaneous use of chicken and trout red blood cells as internal reference standards. Cytometry 3:328-331, 1983

31. Hedley DW. Flow cytometry using paraffin-embedded tissue: Five years on. Cytometry 10:229-241, 1989

32. Tate EH, Wilder ME, Cram LS, Wharton W. A method for staining 3T3 cell nuclei with propidium iodide in hypotonic solution. Cytometry 4:211-215, 1983

33. Coon JS, Landay AL, Weinstein RS. Biology of disease. Advances in flow cytometry for diagnostic pathology. Lab Invest 57:453-478, 1987

CHAPTER TWO

LITERATURE REVIEW

The hallmarks of flow cytometry are shared with those that were monumental in the development of hematological and cytological techniques. Flow cytometry has evolved over the past 25 years from efforts initially intended to count cells and later to size particles. Its progression into a sophisticated analytical tool for rapidly quantitating multiple chemical and physical properties of the individual cell was a result of numerous attempts aimed at the development of new and improved instrumentation to perform these functions. The relevant literature has been reviewed and a historical approach will be presented to recount those developments.

2.1 FLOW CYTOMETRY FOUNDATIONS: MOLDAVAN

American historians of flow cytometry usually cite a 1934 paper by Moldavan[1] as the first attempt to count cells automatically while in flow. He described an apparatus in which a suspension of red blood cells or neutral red stained yeast was forced through a capillary glass tube on a microscope stage, and each passing cell was registered (i.e. counted) by a photoelectric apparatus attached to the ocular. Subsequent to the problems Moldavan encountered in standardizing the capillary tube, assuring proper focus, maintaining flow and obtaining an appropriately sensitive photoelectric apparatus, he did not report any further work.

2.2 THEORY OF HYDRODYNAMIC FOCUSING

Problems with flow in the narrow channels required for flow cytometry and frequent obstruction by large cells remained an important consideration in the

design of later instruments to count cells. A solution to many problems came from the laminar sheath flow principle which was applied by Crosland-Taylor in 1953 to the design of a chamber for optical counting of red blood cells[2]. An aqueous suspension of cells was injected slowly into a faster flowing stream of fluid; the latter provided a laminar sheath surrounding and aligning the particles. This permitted wide-diameter channels to be used with a narrow central stream of the particles to be measured and thus established the basis for hydrodynamic focusing. Almost all flow cytometry instruments today make use of the sheath-flow principle described by Crosland-Taylor.

2.3 THE COULTER ORIFICE

During the early 1950s, several industrial organizations attempted to develop similar blood cell apparatus. One electrical engineer pursuing this goal, Wallace Coulter, encountered problems with the optics and explored another means of cell detection based upon the differences in electrical conductivity between the particles and their suspending medium[3]. Coulter reasoned that blood cells, suspended in a saline solution and passing one at a time through a small orifice, would be detectable by the change produced in electrical impedance of the orifice as they passed through. Based on this principle, a commercially successful instrument for counting blood cells was developed ("Model A" Coulter Counter). During the 1960s, Coulter Counters gradually replaced manual methods of blood cell counting and instruments based on this early principle are now used worldwide in clinical and research laboratories.

2.4 CYTOLOGY FOUNDATIONS: CASPERSSON *ET AL.*

Between the 1930s and the 1960s, the basis for much of modern analytical cytology was established by Torbjorn Caspersson and his colleagues in Stockholm.

Caspersson's 1950 monograph *Cell Growth and Cell Function*[4] describes detailed studies of nucleic acid and protein metabolism during normal and abnormal cell growth which were done by highly precise microspectrophotometric measurements of unstained cells in the ultraviolet and visible regions of the spectrum. By 1950, it was possible to determine the content of nucleic acids and protein in living cells by making measurements near 260 nm and 280 nm. It was also known at this time that certain types of cancer cells were characterized by nuclear enlargement and hyperchromicity (due to increased or abnormal DNA), and this lent itself to possible identification by photometric techniques. Hence, Caspersson embarked on the first field trial of flow cytometry for detection of uterine cervical carcinoma making use of his technique of ultraviolet absorption measurement to identify cells with a high content of nucleic acids.

2.5 KAMENTSKY'S APPARATUS

The individual who can be said to have set the pace for the development of flow cytometry as an analytical tool was Louis Kamentsky, who began to study the problem of automating cervical cytology screening during the early 1960s. He established that nucleic acid content and cell size were useful parameters for cervical cell classification and proceeded to build a flow cytometer which measured the first by light absorption at 260 nm and the second by light scattering at 410 nm. Kamentsky's apparatus was capable of measuring up to four simultaneous parameters per cell and displaying this multiparameter flow cytometric data as two-dimensional histograms[5,6]. He was also the first to record and analyze the data by an interfaced computer[7]. In 1967, a fluidic cell sorter was added to the original apparatus to allow selected cells to be removed for examination by a pathologist, permitting verification of the instrument's performance. By 1970, optical flow

cytometers became available for research purposes when Louis Kamentsky began producing the Cytograf and Cytofluorograf.

2.6 DNA AND PROTEIN QUANTITATION

Although Caspersson has shown that nucleic acids could be quantitated by ultraviolet absorption, UV measurements could not distinguish DNA from RNA. Subsequently, Kamentsky *et al.* described a technique for simultaneous measurements of DNA and protein per cell based on the absorption at two different wavelengths of specifically stained cells[8]. They used the Feulgen reaction for DNA and counterstained with Naphthol Yellow S for protein; then measured absorption at 570 μm (Feulgen) and 430 μm (Naphthol Yellow S). The measurements were displayed as a two-dimensional histogram or as a one-dimensional histogram of the ratio of the two measurements.

2.7 FLUORESCENT STAINING: GOHDE AND DITTRICH

During the mid 1960s, Gohde and Dittrich performed the first simultaneous staining of DNA and protein with fluorescent dyes[9]. They stained DNA with the fluorescent dye ethidium bromide and counterstained for protein with the fluorescent dye 1-dimethylamino-naphthalin-S-sulfonochloride (DANS) or fluorescein isothiocyanate (FITC). They then measured the ethidium bromide fluorescence at wavelengths higher than 590 nm and DANS or FITC stained protein fluorescence simultaneously at 520 or 510 nm, respectively, to obtain a two-parameter analysis of DNA and protein content per cell.

2.8 THE 1960's: THE FLUORESCENCE ERA

Fluorescent dyes provided important advantages over absorbing dyes in the case of flow cytometry systems. When properly used, they eliminated distributional

error, and greatly increased the signal-to-noise ratio (true versus background fluorescence). Fluorescent measurements quickly replaced absorption measurements because of the linear relationship observed between fluorescence signal intensities and quantities of various constituents present in cells. Also, artifacts due to light scattering could more readily be eliminated from fluorescence measurements using optical filters. By the late 1960s, fluorescent stains were in widespread use. In 1967, Kamentsky *et al.* reported three-parameter measurements of acridine orange stained cells - fluorescence at two different wavelength bands and absorption at a third wavelength[10]. At the same time, Van Dilla *et al.* described fluorescent Feulgen measurements of human leucocytes and Chinese hamster ovary cells[11] and Gohde and Dittrich continued to report measurements of cellular DNA by quantitating the fluorescence of ethidium bromide stained cells[9]. This rapidly evolving era made it necessary to develop new fluorochromes and flow cytometric methods that would provide additional information relating to the structure, function and quantity of cellular constituents.

2.9 EARLY FLOW SYSTEMS APPROACH: VAN DILLA

Van Dilla anticipated the extension of fluorescence flow cytometry to work with fluorescent antibodies, to detect fluorescence at multiple wavelengths, and to perform multiparameter analysis using a combination of fluorescence, Coulter volume, and light scattering measurements. During this time, the application of Coons' fluorescent antibody technique[12] to immunologically mark cells containing specific protein variants probably did more to revolutionize flow cytometry than did any other development. Fluorescent-labelled antibodies vaulted the field into adolescence. Briefly, Coons and colleagues originally labelled antibodies with anthracene and could detect the UV - excited blue fluorescence of this label when bound to specimens. In 1950, Coons and Kaplan reported that fluorescein,

conjugated as the isocyanate, gave better results than did anthracene because the blue-excited yellow-green fluorescence was easier to discriminate from auto-fluorescence[13]. As a result of their investigations, fluorescein became, and still remains, the most widely used label in immunofluorescence studies. With the availability of new fluorescent dyes such as fluorescein, and their potential applications in biology and medicine, Van Dilla *et al.* developed a high-speed flow system in 1969 for the quantitative determination of fluorescent light emission from cells containing fluorochrome[11]. Van Dilla's instrument was also the first to have an orthogonal axes of flow, illumination and detection. Their system, used a laminar flow chamber of the Crosland-Taylor design and introduced the argon-ion laser as a light source. Like the sheath-flow principle of Crosland-Taylor, most single-beam flow cytometers today make use of an argon-ion laser as their excitation source.

2.10 MEASUREMENT OF DNA FLUORESCENCE

During their efforts to promote the development of fluorescence, Van Dilla's group was the first to demonstrate a linear relationship between DNA ploidy and the fluorescence intensity of cells stained by a fluorescent Feulgen reaction[11]. They also were the first to produce DNA histograms that clearly defined G_1, S and G_2+M phases of the cell cycle and to demonstrate quantitative cell kinetics with synchronized cultured cells[11]. Today, the measurements of DNA ploidy and cell cycle kinetics have emerged as important applications of flow cytometry.

2.11 FLOW SORTING SYSTEMS

Modifications in the basic design of flow systems led to the sorting capability of instruments. The first working cell sorter was reported in 1965 by Mack Fulwyler who described a device for separating cells based upon electronic cell volume[14].

He adapted the ink jet droplet principle of Richard Sweet[15] using electrostatic deflection of charged droplets as a cell sorting mechanism and was able to sort cells according to their Coulter volume. By 1967, Fulwyler and his colleagues had successfully prepared highly (>95%) purified suspensions of blood granulocytes and lymphocytes[16]. Working independently and at the same time, Kamentsky filed a patent for a device to separate cells in flow after photometric or electrical sensing, using either pneumatic, hydraulic, or electrostatic techniques[17]. Another fluidic switch cell sorter, patented by Friedman[18], diverted the cell stream by means of a sonic transducer that converted laminar to turbulent flow. However, the fastest and most efficient of the sorting devices was the electrostatic sorter. It was adapted for the separation of fluorescence stained cells by Hulett *et al.* (1969)[19]. When a fluorescent cell generated a signal of designated amplitude at the photomultiplier, a charging voltage pulse was applied to the stream. The stream broke into droplets at the frequency of a low-power ultrasonic vibration applied to it. Those droplets formed while the charge was applied, including the one containing the fluorescent cell, remained charged after separation from the stream. These were deflected by an electric field between a pair of charged deflection plates and collected in a separate container from that collecting the undeflected droplets. Operation of this sorter was greatly simplified and improved by Bonner, Hulett and Herzenberg in 1972[20]. By carrying out measurements of the cells in the fluid stream in air, after it left the nozzle of the flow chamber but before droplet formation occurred, minimal delay lapsed between cell measurement and droplet charging. This improved version of a cell sorter, which used a powerful argon ion laser instead of an arc lamp source, could also detect the relatively weak fluorescence of cells stained with fluorescein and rhodamine-tagged antibodies. This instrument was later produced commercially as the Becton Dickinson Fluorescence Activated Cell Sorter® (FACS) and has been widely used since.

2.12 DEVELOPMENTS IN FLOW CYTOMETRY SINCE 1980

During the early years of instrument development, the focus of most studies was the enumeration and characterization of blood cells, the identification and classification of immunoreactive lymphocytes, and the identification of cancer cells in clinical cytology specimens. In the decade since, exciting advances now focus on innovative methods of cell preparation, new fluorescent dyes and unique markers of cellular constituents. These advances have led to an ever-widening spectrum of biological and clinical applications of flow cytometry.

2.13 IMMUNOLOGICAL MEASUREMENTS

Almost from the beginning, flow cytometry has had applications in cellular immunology. With the introduction of monoclonal antibody technology by Kohler and Milstein[21], an almost endless series of powerful, highly specific immunologic reagents became available for cell classification and study. These were first applied in flow cytometry by Reinherz *et al.*[22,23,24] to identify and subclassify T-lymphocytes according to differences in cell surface antigens. They are now available to discriminate a bewildering array of mature and immature lymphocytes and other leucocytes.

Loken was the first to describe the simultaneous measurement of two antigens per cell using fluorescent antibody labels and a single laser for excitation[25]. He corrected electronically for the partially overlapping emission spectra of the two dyes used as fluorescent antibody labels (fluorescein and tetramethyl rhodamine) and enabled dual antigen labelling to be performed.

In 1982, Oi and co-workers made a major contribution to flow cytometry by providing algae phycobiliproteins as fluorescent labels for antibodies and other molecules[26]. Phycoerythrin, a member of the family of phycobiliproteins, contains

more than 20 chromophores per molecule and is extremely efficient at converting absorbed photons to emitted light (quantum yield = 0.98). The fluorochrome is excited with an argon laser (488 nm) and emits an orange fluorescence readily separable from the green fluorescence of fluorescein. Thus, phycoerythrin labelled monoclonal antibodies, which are now available commercially, have made dual-label immunofluorescence flow cytometry practical with single-beam argon laser instruments.

2.14 PHYSIOLOGICAL MEASUREMENTS

The applications of flow cytometry have extended beyond immunologically identifying surface antigens to include numerous physiological measurements. In 1985, a new fluorescent dye, Indo 1, introduced by Grynkiewicz *et al.*[27] made practical the precise measurement of intracellular calcium ion concentration by flow cytometry. The dye is excited by ultraviolet light and exhibits a large fluorescence emission wavelength shift in the presence of free calcium ion (485 nm) versus calcium complex (410 nm). The ratio of fluorescence intensities at the two different emission wavelength bands permits calculation of intracellular calcium ion concentration independent of dye concentration. Since changes in calcium ion concentration are an early marker in cell activation, this dye can be used to identify and study the process by flow cytometry. Other physiologic measurements of cell function by flow cytometry include changes in intracellular pH first described by Visser *et al.*[28], cell surface charge describing initially by Valet *et al.*[29] and redox state described by Thorell[30].

2.15 HEMATOLOGICAL MEASUREMENTS

Among the most important clinical applications of flow cytometry are those in hematology. Within a few years, flow cytometry has become the standard method

of blood cell counting, including the differential counting of leucocytes and, with monoclonal antibodies, the subclassification of lymphocytes. Tanke *et al.* expanded this repertoire of measurements to include red blood cell reticulocyte counts based on RNA measurements[31].

2.16 DNA FLOW CYTOMETRY TODAY

One of the first, and most widely used, applications of flow cytometry is in measuring the DNA content of cells from solid tumors. Many but not all tumors contain populations of measurably aneuploid cells, and much controversy still centers on the possible significance of DNA aneuploidy. In 1983, a pivotal paper in the history of DNA flow cytometry was published. Hedley and associates described a technique for DNA flow cytometry of tumor cell nuclei extracted from formalin-fixed tissues embedded in paraffin blocks[32]. Its impact on our understanding of the value of DNA analysis has been considerable. It has taken us from an era in which only fresh samples could be analyzed, which meant prospective studies with small patient numbers and limited follow-up, to one in which whole pathology archives are available for retrospective analysis. Such a technique offers hope that the true significance of DNA aneuploidy in solid tumors will be firmly established following completion of these retrospective studies.

2.17 FUTURE DIRECTIONS OF FLOW CYTOMETRY

These wide ranging applications of flow cytometry ushered in a still continuing period of staining and techniques development. The methodology continues to improve to match the state-of-the-art, highly sophisticated engineering of modern day flow cytometers. Soon multiparameter analysis will go far beyond the current capabilities, and better probes and stains will allow more precise

analysis of cellular characteristics enabling applications that now seem esoteric to become routine.

REFERENCES

1. Moldvan A. Photo-electric technique for the counting of microscopic cells. Science 80:188-189, 1934

2. Crosland-Taylor PJ. A device for counting small particles suspended in a fluid through a tube. Nature 171:37-38, 1953

3. Coulter WH. Highspeed automatic blood cell counter and cell size analyzer. Proc Natl Electron Conf 12:1034-1042, 1956

4. Caspersson T. Cell growth and cell function. Norton, New York, 1950

5. Kamentsky LA, Melamed MR, Derman H. Spectrophotometer: New instrument for ultrarapid cell analysis. Science 150:630-631, 1965

6. Kamentsky LA. Rapid biological cell identification by spectroscopic analysis. Proc 18th Ann Conf on Engineering in Biol and Med 7:178, 1965

7. Kamentsky LA, Melamed MR. Instrumentation for automated examination of cellular specimens. IEEE 57:2007-2016, 1969

8. Kamentsky LA, Thorell B. Cell population identification studies. Acta Cytol 14:307-312, 1970

9. Dittrich W, Gohde W, Severin. Die Kern-Plasma-Relation in Der Impulscytophotometrie Des Cervical-Und Vaginalsmears. 4th Int'l Cong of Cytol, London (Abstract) 1971

10. Kamentsky LA, Melamed MR. Rapid multiple mass constituent analysis of biological cells. Ann N Y Acad Sci 157:310-323, 1969

11. Van Dilla MA, Trujillo TT, Mullaney PF, Coulter JR. Cell microfluorometry: A method for rapid fluorescence measurement. Science 163:1213-1214, 1969

12. Coons AH, Creech HJ, Jones RN. Immunological properties of an antibody containing a fluorescent group. Proc Soc Exptl Biol Med 47:200-202, 1941

13. Coons AH, Kaplan MH. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J Exp Med 91:1-14, 1950

14. Fulwyler MJ. Electronic separation of biological cells by volume. Science 150:910-911, 1965

15. Sweet RG. High frequency recording with electrostatically deflected ink jets. Rev Sci Instr 36:131-136, 1965

16. Van Dilla MA, Fulwyler MJ, Boone IU. Volume distribution separation of normal human leucocytes. Proc Soc Exptl Biol Med 125:367-374, 1967

17. Kamentsky LA. U.S. Patent No. 3,560,754. Photoelectric particle separator using time delay, Filed November 17, 1965 Issued February 12, 1971

18. Friedman M. U.S. Patent No. 5,791,517. Digital fluidic amplifier particle sorter, Filed March 5, 1973 Issued February 12, 1974

19. Hulett HR, Bonner WA, Barrett J, Herzenberg LA. Cell sorting: Automated separation of mammalian cells as a function of intracellular fluorescence. Science 166:747-749, 1969

20. Bonner WA, Hulett HR, Sweet RG, Herzenberg LA. Fluorescence activated cell sorting. Rev Sci Instr 43:404-409, 1972

21. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-497, 1975

22. Kung PC, Goldstein G, Reinherz EL, Schlossman SF. Monoclonal antibodies defining distinctive human T cell surface antigen. Science 206:347-349, 1979

23. Reinherz EL, Kung PC, Goldstein G, Schlossman SF. A monoclonal antibody with selective reactivity with functionally mature thymocytes and all peripheral human T cells. J Immunol 123:1312-1317, 1979

24. Reinherz EL, Kung PC, Goldstein G, Schlossman SF. Separation of functional subsets of human T cells by a monoclonal antibody. Proc Natl Acad Sci 76:4061-4065, 1979

25. Loken MR, Parks DR, Herzenberg LA. Two color immunofluorescence using a fluorescence activated cell sorter. J Histochem Cytochem 25:899-907, 1977

26. Oi VT, Glazer AN, Stryer L. Fluorescent phycobiliprotein conjugates for analysis of cells and molecules. J Cell Biol 93:981-986, 1982

27. Grynkiewicz G, Poenie M, Tsien Ry. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440-3450, 1985

28. Visser JWM, Jongeling AAM, Tanke HJ. Intracellular pH determination by fluorescence measurements. J Histochem Cytochem 27:32-35, 1979

29. Valet G, Bamberger S, Hoffmann H, Schindler R, Ruhenstroth-Bauer G. Flow cytometry as a new method for the measurement of electrophoretic mobility of erythrocytes using membrane charge staining by fluoresceinated polycations. J Histochem Cytochem 27:342-349, 1979

30. Thorell B. Intracellular red-ox steady states as basis for characterization by flow cytofluorometry. Blood Cells 6:745-751, 1980

31. Tanke HJ, Nieuwenhuis IA, Koper GJ, Slats JC, Ploem JS. Flow cytometry of human reticulocytes based on RNA fluorescence. Cytometry 1:313-320, 1981

32. Hedley DW, Friedlander ML, Taylor IW *et al.* Method of analysis of cellular DNA content of paraffin-embedded pathological material using flow cyometry. J Histochem Cytochem 31:1333-1335, 1983

CHAPTER THREE

AUTOMATED PROCEDURE FOR DEWAXING AND REHYDRATION OF PARAFFIN-EMBEDDED TISSUE SECTIONS FOR FLOW CYTOMETRIC DNA ANALYSIS OF BREAST TUMORS*

3.1 INTRODUCTION

Flow cytometric analysis of DNA content in single cells rapidly and accurately determines the cell cycle distribution and ploidy. Hedley and associates[1] recently developed a technique that allows the flow cytometric quantitation of cellular DNA content in previously paraffin-embedded pathologic material. This method has permitted numerous retrospective studies evaluating the presence or absence of DNA-aneuploid stemlines and cell cycle distribution. The study of paraffin-embedded tissues still has many limitations: (1) the time-consuming and laborious multistep procedure for dewaxing and rehydrating tissues; (2) the inability to use routine internal standards in formalin-fixed tissue; (3) the effect of tissue fixation on ploidy analysis and (4) relatively poor resolution compared with that of fresh material. To improve this technique, we developed an automated procedure to complete the dewaxing and rehydration of tissue while it is in a single processing and embedding cassette. In this chapter, we report the efficiency of this automated technique in a study of paraffin-embedded breast cancer tissue.

3.2 MATERIALS AND METHODS

3.2.1 Tissue Preparation

Ninety-three samples from 69 patients were analyzed for cellular DNA content. The patients' slides were reviewed, and, where possible, at least two representative blocks of normal and malignant paraffin-embedded tissues were

* A version of this chapter has been published. Babiak JC and Poppema S. Automated Procedure for Dewaxing and Rehydration of Paraffin-Embedded Tissue Sections for Flow Cytometric DNA Analysis of Breast Tumors. Am J Path 96:64-69, 1991

selected for DNA flow cytometry. The appropriate blocks were retrieved, and three 50-μm sections were cut from each. Occasionally, more sections were required if the tissue block was small. To determine the tissue representation and confirm the original diagnosis, a routine 5-μm section was also cut after each series of 50-μm sections and stained with hematoxylin and eosin.

Before all 93 samples were analyzed, the manual and automated dewaxing and rehydrating methods were compared side by side, using an identical set of samples (five breast carcinomas, one lymph node), to determine the reproducibility of the two techniques. Briefly, for the manual method, a modification of the procedure of Hedley and associates[1] was used. Tissue sections were placed in glass centrifuge tubes and dewaxed with three changes of xylene (3 mL for 10 minutes at room temperature) and rehydrated in a series of ethyl alcohol strengths (3 mL of 100, 95, 70 and 50% for 10 minutes each) at room temperature. The tissue was then washed in distilled water twice. In the automated method, each set of sections was placed in an aspiration bag (Shandon Inc., Pittsburgh, PA, USA) and enclosed in a processing and embedding cassette. The cassettes were placed in a metal cassette carrier that holds as many as 30 cassettes.

The tissue was dewaxed with three changes of xylene and rehydrated in the same series of graded ethanols used in the manual method, followed by two final washes in distilled water. The solutions were kept in 1-L volumetric beakers and arranged sequentially on a tissue-processing machine (Sakura Fine Technical Company, Ltd., Tokyo, Japan) (Figure 3.1). The entire multistep dewaxing and rehydrating procedure was performed automatically on the tissue processor, as the cassette carrier rotated sequentially from one solution to the next.

The processor was equipped with a 24-hour automatic timer, yielding a minimum incubation interval of 30 minutes in each solution. To initiate the automated dewaxing and dehydration, samples were first placed in a dry beaker with

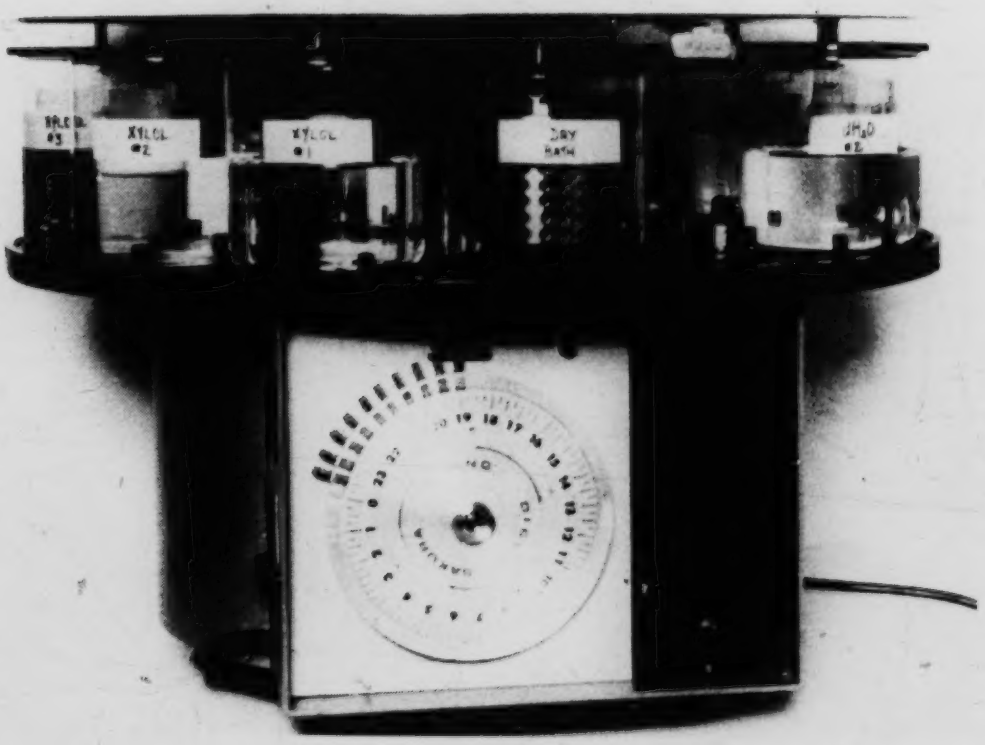


FIGURE 3.1

Routine histological processor equipped with a 24-hour timer to automatically commence dewaxing and rehydration of paraffin-embedded tissue. A metal cassette carrier is used to hold multiple processing and embedding cassettes. Volumetric beakers (1,000 mL) contain the solutions through which the cassette carrier sequentially rotates.

the timer set to rotate the cassette carrier into the first dewaxing solution within 15 minutes. Once the carrier passed through all the required solutions, it remained in the last beaker of distilled water until it was removed from the processor. Uniform mixing was ensured throughout the procedure as the tissue processor continuously rotated the cassette carrier from left to right. After the final wash in distilled water, the cassette carrier was removed and each cassette was opened. The tissue was removed from the aspiration bags with fine-tip forceps, placed in 1.5-mL polypropylene Eppendorf tubes (Brinkmann Instrument Company, Westbury, NY, USA) and minced with straight scissors while immersed in distilled water. Tissue that was dewaxed and rehydrated manually was minced and digested enzymatically in the same manner as that which was prepared automatically.

The tissue was transferred to 15-mL tubes, centrifuged (all centrifugations were for 10 minutes at 500 X *g*, 4°C), and resuspended in 1 mL of 0.5% [weight/volume(w/v)] pepsin (Sigma Chemical Company, St. Louis, MO, USA) in 0.9% (w/v) NaCl (adjusted to pH 1.5 with 2 N HCl) containing 3% (w/v) polyethylene glycol (PEG) 6000. The tubes were placed in a 37°C water bath for 30 minutes, with brief vortexing at 5-minute intervals. The recovered nuclei were then filtered through a 52-μm nylon mesh (Spectrum Medical Industries, Inc., Los Angeles, CA, USA) into a clean 15-mL centrifuge tube, and the pH was neutralized by increasing the volume to 5 mL with cold 25 mmol/L HEPES-Hank's balanced salt solution (HBSS) containing 3% (w/v) PEG 6000 and 0.02% (w/v) azide. The suspensions were centrifuged, and the nuclear pellets were resuspended in 2 mL HEPES-HBSS. An aliquot (50 μl) was removed for determining nuclear concentration using the Trypan Blue method. A final concentration of 1-2 x 10^6 nuclei/ml was used for DNA staining. It was also found that samples could be stored in HEPES-HBSS for several days at 4°C without changes in the DNA staining pattern.

3.2.2 DNA Staining

The suspensions were centrifuged and resuspended in 0.5% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), at a concentration of 1-2 X 10^6 nuclei/mL, for 10 minutes at 4°C. After another centrifugation, 1 mL of cold 0.1% (v/v) Triton X-100 (Sigma Chemical Company, St. Louis, MO, USA) in PBS was added to the samples and left to incubate on ice for 3 minutes, to render the nuclei permeable to the staining fluorochrome. Again, the nuclei were centrifuged and resuspended in RNAse (180 units/mL in PBS) for 30 minutes at 37°C to remove residual RNA. After centrifugation, the nuclei were stained with 50-μg/mL propidium iodide (PI) (Sigma) in PBS. All PI stained samples incubated for at least 30 minutes before flow cytometric analysis.

3.2.3 Flow Cytometric DNA Analysis

The stained nuclear suspensions were analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) with a 488 nm argon laser as an excitation source. Instrument calibration and performance were monitored with CaliBRITE beads and the AUTOCOMP software designed by Becton Dickinson Immunocytometry Systems. Information regarding instrument linearity and resolution was obtained by analyzing ethanol-fixed chicken erythrocyte nuclei. Before flow analysis, each sample was passed several times through a 25 gauge needle with a syringe and filtered through a 52-μm nylon mesh filter. At least 20,000 events were measured per sample, and low flow rates (12 μL/minute) were used to maximize resolution. The DNA index (DI) and cell cycle distribution were determined with the CELLFIT software developed by Becton Dickinson.

3.3 RESULTS

After comparison of the results of the same set of samples (five carcinomas, one lymph node) that were prepared manually and automatically, no significant differences were identified. The coefficients of variation (CVs) were comparable in both sets, no loss of aneuploid populations was detected, and the DNA staining patterns were unremarkable as judged by the reproducible histograms (Figure 3.2).

After the two methods were compared side by side, 93 specimens from 69 node-negative breast cancer patients were prepared automatically and evaluated. The tissues included 24 non-involved lymph nodes and 69 breast carcinomas of various types. Only one tumor sample was rejected because of significant baseline debris and an uninterpretable histogram. Of the remaining 68 carcinomas, the quality of the DNA histograms was acceptable in 90% (61 of 68) of the samples analyzed. Although appearing diploid, seven tumor samples has significantly greater and unacceptable CVs (8.3-16.2). (In this study, the CV of the G_0/G_1 peak width of all tumors had to be less than 6.0 for the specimen to be considered interpretable for ploidy status.)

Although a reliable standard is not available for use with paraffin-embedded material, 24 non-involved lymph nodes, resected at the time of surgery and fixed in the same manner as the tumor samples, were available as diploid standards. In these tumor samples, most (20 of 24) G_0/G_1 peaks coincided with the peak that was defined as diploid in the lymph nodes. The remaining four tumors had diploid G_0/G_1 peaks that did not coincide with the G_0/G_1 channel number of the defined normal population in the lymph nodes. The DI was determined according to a recently suggested convention on nomenclature for DNA cytometry[2]. DI was defined as the ratio of the mean or mode of the relative DNA content of the G_0/G_1 cells of the sample divided by the mean or mode of the relative DNA measurement of the diploid G_0/G_1 reference cells.

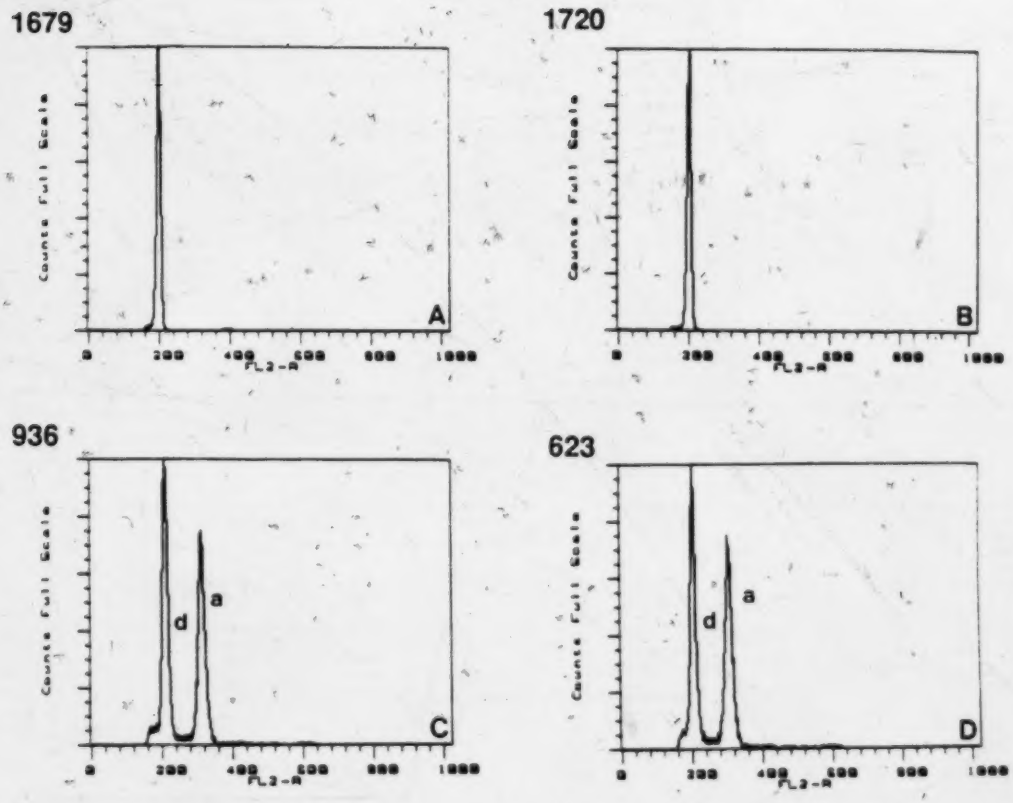


FIGURE 3.2

Side-by-side comparison of the DNA histograms from the same set of samples prepared manually and automatically. The number of particles is given on the vertical axis and DNA content (channel numbers) on the horizontal axis. Coefficient of variation (CV) of the G_0/G_1 peak of the lymph node prepared (A) manually and (B) automatically was 2.8 and 2.9, respectively. CVs of the diploid populations of the tumor prepared (C) manually and (D) automatically were 3.6 and 3.3, respectively. Under the same conditions, the CVs of the aneuploid G_0/G_1 peaks were 2.9 and 3.4, respectively. DNA indices of the tumor were similar: manual DI = 1.49 and automated DI = 1.48.

For the paraffin-embedded samples, the first peak in the histogram was considered as a normal diploid standard and a DNA histogram was said to contain a DNA aneuploid population only when two discrete G_0/G_1 peaks were demonstrated. Of the 61 tumor samples examined, 25 (41%) were diploid and 36 (59%) were aneuploid. The distribution of DIs ranged from 1.08 to 2.17. The mean CVs for the G_0/G_1 peak of the diploid and aneuploid tumors were 4.54 and 4.18, respectively. Representative DNA histograms from paraffin-embedded tissue that were dewaxed and rehydrated automatically are shown in Figure 3.3.

3.4 DISCUSSION

The development of a method for flow cytometric DNA analysis of routinely prepared paraffin-embedded tissue by Hedley and colleagues[1] has permitted numerous retrospective clinical studies. The method also allows rare, but clinically important, tumors to be studied because samples can be pooled from several institutions. Moreover, it permits assessments of DNA content as an independent prognostic variable in patients whose clinical outcome is known.

The paraffin technique is long and tedious, and we have modified it to reduce the laborious multistep procedure for dewaxing and rehydrating tissue. Sickle-Santanello and associates[3] demonstrated the use of a specially designed container to hold multiple processing and embedding cassettes to minimize the time required for individual samples to be dewaxed and rehydrated.

Herein, we report a technical improvement that automates this entire step by using a routine histological tissue processor. This method reduces the amount of technologist time required to perform this laborious step because the timer on the processor can be set to start the automatic dewaxing and rehydration process. When the process is started after laboratory hours, samples can dewax and rehydrate

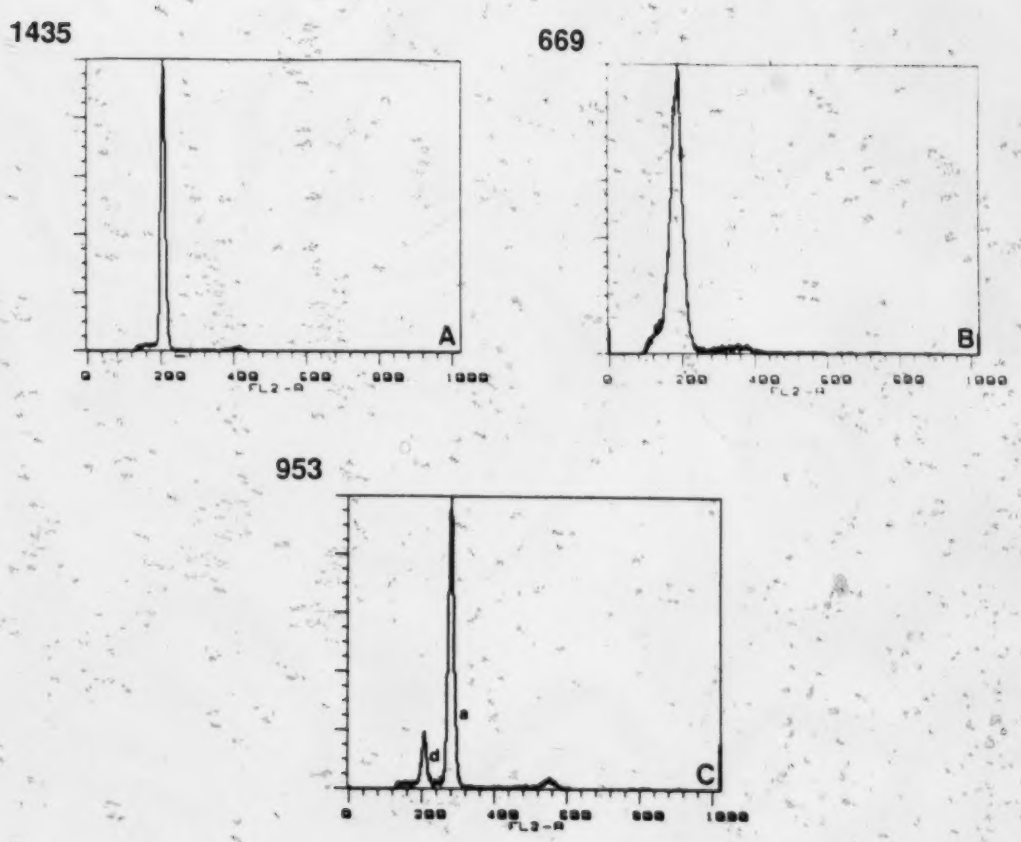


FIGURE 3.3

Examples of DNA histograms prepared by the automatic dewaxing and rehydration procedure. A. Diploid tumor, 2.8. B. Unacceptable DNA distribution of a diploid tumor with a wide coefficient of variation. CV 9.7. C. Aneuploid tumor, DNA index 1.35. CVs of the G_0/G_1 peaks of the diploid and aneuploid populations were 2.5 and 3.3, respectively.

overnight and be available early the next day for enzymatic digestion. Although the tissue samples incubate in each solution for 30 minutes, rather than the previously suggested 10 minutes, this has no effect on the reproducibility or the quality of DNA staining. We also found that tissue recovery was excellent, and the performance of all steps while the tissue was in an aspiration bag enclosed in a single cassette minimized the chance of losing or fragmenting the tissue. In addition, because more samples can be batched during a single run, the efficiency of this technique is increased; the cassette carrier can hold as many as 30 cassettes.

Ninety-three samples (69 tumors, 24 uninvolved lymph nodes) were prepared automatically after a side-by-side comparison of the two methods. There were no significant differences in the same set of samples: the CVs, recovery of aneuploid populations, and DNA staining patterns were all comparable. The 59% frequency of DNA aneuploidy for the breast carcinomas in this study agrees with a number of previous reports.[4-9] In four tumor samples, the G_0/G_1 peak did not coincide with the external diploid cells, possibly because of differences in fixation that we cannot control with the use of archival specimens. Seven tumor samples with higher CVs (8.3-16.2) were excluded from our analysis because of the risk of overlooking near-diploid tumors. Hedley and colleagues suggested that a greater proportion of paraffin-embedded tumors have a DI of 1.0, but this is associated with fewer near-diploid tumors (i.e. DI < 1.0-1.1) when DNA analysis is performed on both fresh and paraffin-embedded material.[10] Hence, the percentage of diploid tumors reported in this study may be underestimated since the full extent to which an increasing CV leads to a loss of near-diploid G_0/G_1 peaks remains uncertain.

In conclusion, the technical improvement we have described in this study yields reproducible results, is more efficient than previously suggested methods, and opens the way for other advances to further improve the paraffin-embedded technique for DNA flow cytometric analysis and to reduce its current limitations.

REFERENCES

1. Hedley DW, Friedlander ML, Taylor IW, *et al.* Method of analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 31:1333-1135, 1983

2. Hiddemann W, Schumann J, Andreeff M, *et al.* Convention on nomenclature for DNA cytometry. Cytometry 5:445-446, 1984

3. Sickle-Santanello BJ, Farrar WB, DeCenzo JF, *et al.* Technical and statistical improvements for flow cytometric DNA analysis of paraffin-embedded tissue. Cytometry 9:594-599, 1988

4. Hedley DW, Rugg CA, Ng AB, Taylor IW. Influence of cellular DNA content on disease-free survival of stage II breast cancer patients. Cancer Res 44:5395-5398, 1984

5. Kallioniemi O-P, Blanco G, Alavaikko M, *et al.* Tumor DNA ploidy as an independent prognostic factor in breast cancer. Br J Cancer 56:637-642, 1987

6. Kallioniemi O-P, Blanco G, Alavaikko M, *et al.* Improving the prognostic value of DNA flow cytometry in breast cancer by combining DNA index and S-phase fraction. Cancer 62:2183-2190, 1988

7. Muss HB, Kute TE, Case LD, *et al.* The relation of flow cytometry to clinical and biologic characteristics in women with node-negative primary breast cancer. Cancer 64:1984-1900, 1989

8. Owainati AA, Robins RA, Hinton C. Tumor aneuploidy, prognostic parameters and survival in primary breast cancer. Br J Cancer 55:449-454, 1987

9. Thorud E, Fossa SD, Vaage S, *et al.* Primary breast cancer. Flow cytometric DNA pattern in relation to clinical and histopathologic characteristics. Cancer 57:808-811, 1986

10. Hedley DW, Friedlander ML, Taylor IW. Application of DNA flow cytometry to paraffin-embedded archival material for the study of aneuploidy and its clinical significance. Cytometry 6:327-333, 1985

CHAPTER FOUR

SIGNIFICANCE OF c-*erb*B-2 AMPLIFICATION AND DNA ANEUPLOIDY: AN ANALYSIS IN 78 NODE-NEGATIVE BREAST CANCER PATIENTS*

4.1 INTRODUCTION

Breast cancer continues to be one of the leading causes of cancer deaths among women. Approximately 50-60% of patients with breast cancer have disease that is confined to the breast without axillary nodal involvement[1]. Although the great majority (70%) of these patients will enjoy long-term survival following local surgery, 30% of axillary node-negative (ANN) patients will have a recurrence of their disease[2,3]. This latter group would benefit from adjuvant therapy[4-7]; however, physicians are reluctant to administer treatment because of the difficulty identifying that minority of patients who will relapse. Many current prognostic factors, including tumor size, histologic and nuclear grade and hormone receptor status do not clearly separate ANN patients into low- and high-risk groups. In view of the present limitations of conventional prognostic factors, additional second-generation determinants would be valuable in selecting those patients most likely to benefit from adjuvant therapy. This chapter describes our investigations into the determinations of c-*erb*B-2 proto-oncogene amplification and cellular DNA content in primary breast cancer patients without axillary node involvement.

4.2 MATERIALS AND METHODS

A total of 704 female subjects diagnosed as having invasive carcinoma of the breast with negative axillary lymph nodes were registered in the Breast Unit of the Cross Cancer Institute during the 12-year period from 1971 to 1982 inclusive. This cohort formed the population base of our study. From this base, 115 patients

* A version of this chapter has been accepted: Babiak JC, Hugh J and Poppema S. Significance of c-erbB-2 Amplification and DNA Aneuploidy: An Analysis in 78 Node-Negative Breast Cancer Patients. Cancer, September, 1991

(cases) who had relapsed (i.e., developed locoregional recurrence and/or distant metastasis after initial surgery) were matched pairwise with 115 controls who had not as yet recurred during the same follow-up interval. Pairwise case-control matches were made by the following criteria: size of primary tumor at clinical examination; menopausal status; estrogen receptor (ER) status; anniversary year of initial treatment (± 3 years) and age at treatment (± 5 years). Proto-oncogene copy number was previously determined on all 230 tumor tissue blocks. Of these 230 patients, 78 (41 cases, 37 controls) were selected for flow cytometric DNA analysis, the remaining tumors being insufficient for analysis or unavailable. The study was kept blinded as to whether the tumor blocks were derived from cases or controls until completion of the measurements of c-*erb*B-2 copy number and DNA ploidy. Forty of the 78 patients were matched for the five clinicopathological criteria with the remaining 38 being concordant in at least three of the selection variables. Treatment regimens were essentially identical for the two groups. The vast majority of patients were treated for their primary tumor by either radical or total mastectomy and sampling of the lower axillary lymph nodes. No patient received any form of treatment prior to surgery. The mean number of nodes examined in both cases and controls was nine, ranging from 4 to 25 and 2 to 24, respectively. Absence of axillary node disease was determined by histological examination of lymph nodes resected at the time of primary treatment.

4.2.1 Determination of c-*erb*B-2 Copy Number

All available formalin-fixed, paraffin-embedded tumor blocks were retrieved. A routine histological slide was prepared from each block and a representative section of tumor was chosen by a single reference pathologist. Proto-oncogene (c-*erb*B-2) copy number was determined from the selected block by slot-blot hybridization as previously described[8]. Two non-established dermal fibroblast

strains (GM 38 and GM 43) served as standards for the determination of tumor DNA samples of normal diploid constitution (i.e., those said by convention to contain a single copy of a particular gene). Both strains were obtained from the NIGMS Human Genetic Cell Repository, Camden, NJ, USA. The number of copies of c-*erb*B-2 was estimated for individual tumor specimens by comparing the signal intensity of serially diluted DNA blots of a given specimen with the average of that found (taken as the single copy value) for the two normal fibroblast strains. A tumor specimen was categorized as amplified if the extracted DNA was found to contain at least three genomic equivalents of the c-*erb*B-2 proto-oncogene. The presence of a normal copy number of the gene encoding the tumor antigen p53 in the tumor DNA samples excluded increased ploidy of chromosome 17 as a possible explanation for the observed amplification of c-*erb*B-2. The results of this analysis are reported separately in greater detail by Paterson *et al.*[9].

4.2.2 Flow Cytometric DNA Analysis

Paraffin-embedded tissues were prepared for flow cytometric DNA analysis using a modification of the Hedley technique[10]. A published automated procedure for dewaxing and rehydrating the tissue was used to reduce the amount of time required to prepare the samples[11]. Briefly, 50-μm sections were cut from the paraffin-embedded tumor block and an adjacent 5-μm section was stained with hematoxylin and eosin to determine tissue representation. The sections were placed in aspiration bags and enclosed in processing and embedding cassettes. The casettes were placed in a metal cassette carrier which rotated sequentially through a series of xylenes and graded alcohols on a routine histological tissue processor (Sakura Fine Technical Co. Ltd., Tokyo, Japan). After the final wash in distilled water, nuclear suspensions from dewaxed, rehydrated tissue sections were prepared using an acidic pepsin solution and stained stoichiometrically with propidium iodide. The

suspensions were passed through a 25 gauge needle before filtration through a 52-μm pore size nylon mesh. Cellular DNA content was measured using a FACScan® flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and analyzed with Cellfit software. The fluorescence signals of at least 20,000 nuclei were collected and the result displayed as a frequency-distribution histogram (DNA histogram).

The degree of DNA content aberration was expressed by the DNA index. Since a reliable standard deviation is not available for determination of DI in paraffin material, we have assumed that the G_0/G_1 peak with the smallest DNA content represented normal diploid cells. According to the suggested convention on nomenclature for DNA cytometry, a DNA histogram was said to contain an aneuploid population only when two separate G_0/G_1 peaks were observed[12].

The ability to identify additional peaks lying close to the normal population depends on the resolution of the measurement of cellular DNA. This can be expressed as the coefficient of variation (CV) of the G_0/G_1 peak. The greater the CV, the greater the chance of overlooking near-diploid populations. In the present series, the coefficient of variation of the G_0/G_1 peak width of all tumors had to be less than 6.0 for the DNA histogram to be considered interpretable for ploidy status.

4.3 RESULTS

Results of the c-*erb*B-2 copy number and DNA ploidy analysis in the 78 cases and controls are presented in Table 4.1. Choosing a gene copy number of three as the lower limit for amplification, a 3- to 22- fold elevation of c-*erb*B-2 was observed in 10% (8/78) of the tumors. Six of the eight tumors with c-*erb*B-2 elevation were obtained from the case group and the remaining two came from the control group. This indicated that 15% (6/41) of the node-negative patients who had relapsed had

Table 4.1. Results of c-erbB-2 determination and flow cytometric DNA analysis in 78 node-negative primary breast cancer patients.*

	Cases (n=41)		Controls (n=37)	
	non-amplified	amplified	non-amplified	amplified
Diploid	17	2	20	2
Aneuploid	18	4	15	0
Total	35	6	35	2

*c-erbB-2 gene status obtained from data base used in Paterson et al (reference 9)

multiple copies of the proto-oncogene in their malignant tissue compared to 5% (2/37) for the patients who remained disease-free for protracted periods. Eighty-five percent (35/41) of the patients who relapsed harbored normal levels of the proto-oncogene.

DNA histograms revealed that 41 tumors were diploid and 37 were aneuploid. The presence of an abnormal DNA stemline was demonstrated in 22 cases and 15 controls. The distribution of DNA indices ranged from 1.12 - 2.17 with a mean CV for the G_0/G_1 peak of the diploid and aneuploid tumors of 4.6 and 4.0, respectively. Nineteen of 41 patients (46%) with diploid tumors and 22 of 37 patients (59%) with aneuploid tumors developed recurrences. Compared to c-*erb*B-2 amplification, flow cytometric DNA analysis identified a higher number of high-risk patients who relapsed by revealing distinct aneuploid stemlines in their tumors; however, ploidy measurement generated a significantly greater proportion of false positives. From our results, 41% of the controls who did not have a recurrence of their disease during the same follow-up time interval displayed aneuploidy in their tumors. The DNA content in tumors with normal copy numbers of c-*erb*B-2 ranged from diploid to varying degrees of aneuploid (mean DNA index of 1.60). There was also no tendency for amplified tissues to contain a tetraploid DNA content as diploid and aneuploid distributions were demonstrated in these tumors. The DNA indices of the amplified tumors showing aneuploid DNA patterns were 1.55, 1.65, 1.75 and 1.87. Representative DNA histograms from paraffin-embedded tumors containing c-*erb*B-2 amplification are shown in Figure 4.1. The statistical indices of sensitivity and specificity for c-*erb*B-2 determination and DNA ploidy analysis are illustrated in Tables 4.2 and 4.3, respectively.

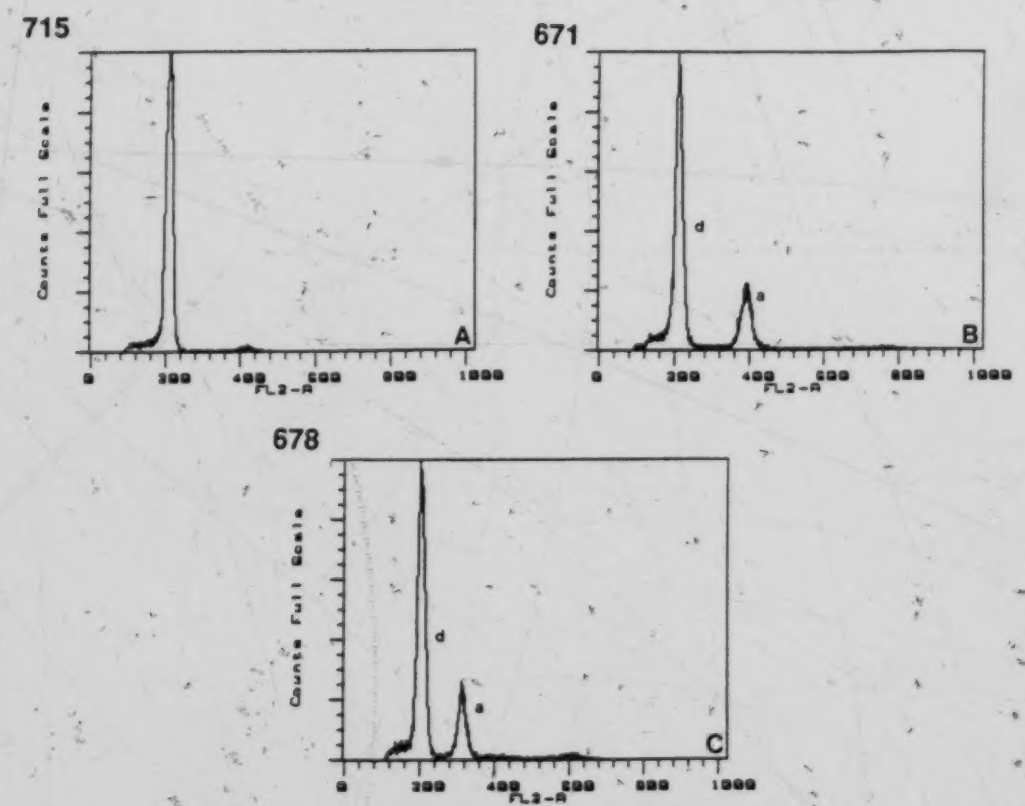


FIGURE 4.1

Representative DNA histograms from paraffin-embedded breast tumors with c-*erb*B-2 amplification. A. Case 22. Diploid DNA distribution with 8-fold elevation. CV of G_0/G_1 peak 4.7. B. Case 114. DNA index 1.87 with 3-fold elevation. CV of diploid and aneuploid G_0/G_1 peaks of 4.4 and 4.0, respectively. C. Case 115. DNA index 1.55 with 15-fold elevation. CV of diploid and aneuploid G_0/G_1 peaks of 5.0 and 3.8, respectively.

Table 4.2. Statistical analysis of c-erbB-2 determination as a predictive test of relapse in node-negative primary breast cancer.*

	Relapse		
	Positive	Negative	
New test: c-erB-2 amplification			
Positive	a 6	b 2	a+b 8
Negative	c 35	d 35	c+d 70
	a+c 41	b+d 37	a+b+c+d 78

* data derived from Table 4.1

a - true positives
b - false positives
c - false negatives
d - true negatives

Sensitivity is defined as the proportion (or percentage) of diseased subjects who have a positive test = $\frac{a}{a+c}$

Specificity is the proportion (or percentage) of disease-free subjects who have a negative test = $\frac{d}{d+b}$

Table 4.3. Statistical analysis of DNA flow cytometry as a predictive test of relapse in node-negative primary breast cancer.*

	Relapse		
	Positive	Negative	
New test: DNA Aneuploidy			
Positive	a 22	b 15	a+b 37
Negative	c 19	d 22	c+d 41
	a+c 41	b+d 37	a+b+c+d 78

* data derived from Table 4.1

a - true positives
b - false positives
c - false negatives
d - true negatives

Sensitivity is defined as the proportion (or percentage) of diseased subjects who have a positive test = $\frac{a}{a+c}$

Specificity is the proportion (or percentage) of disease-free subjects who have a negative test = $\frac{d}{d+b}$

4.4 DISCUSSION

Lymph node-negative patients comprise 50-60% of all newly diagnosed breast cancers[13]. This percentage will probably increase with the implementation of screening programs for early detection. Recognizing the overall favorable prognosis of node-negative breast cancer, the appropriateness of adjuvant therapy in this group of patients is controversial. At present, there are no markers which can be used to reliably identify women who are at high risk for relapse and who would benefit from adjuvant therapy. We evaluated the ability of two second generation prognosticators, including c-*erb*B-2 amplification and DNA ploidy, to identify with greater accuracy this minority of high-risk patients.

Recent attention has been directed to the importance of proto-oncogenes in human malignancies. Proto-oncogenes represent a family of normal cellular genes involved in cell growth and differentiation. There is evidence that alterations either in gene structure, gene copy (amplification) or overexpression may be involved in the pathogensis of some human cancers[14]. The c-*erb*B-2 proto-oncogene, located on band 21 of chromosome 17[15-18], encodes a 185-kd transmembrane protein with tyrosine kinase activity[15-17]. Its homology with the epidermal growth factor receptor (EGFR) suggests it too is a membrane receptor[17]. In a large series of human primary breast cancers, Slamon showed that amplification of the c-*erb*B-2 gene correlated with overall survival and time to relapse[18]. Since that initial report, other studies have been published reporting considerable variation in both the incidence of amplification/overexpression and prognostic significance of c-*erb*B-2. Some groups have found amplification in only 10% of patients and no correlation to clinical outcome[19,20], while others have found an incidence as high as 33% and a strong association with outcome[21-24]. These conflicting observations may be due, in part, to methodologic variability, short-term follow-up and differences in patient cohorts. In the present study, we eliminated this variability by selecting a

homogenous cohort of node-negative patients whose long-term follow-up was known. Moreover, by applying slot-blot hybridization on tumor DNA extracted from formalin-fixed paraffin-embedded tissue, an accurate determination of proto-oncogene number was made.

The magnitude of c-*erb*B-2 gene amplification was determined in 78 patients (41 cases, 37 controls) selected from a cohort of approximately 700 node-negative breast cancer patients with 5-16 years follow-up. Amplification (3- to 22-fold) was present in a higher portion (15 vs 5%) of the tumors from patients who had relapsed (cases) than from those who had not (controls). Using Chi-squared analysis with Yates correction, there was no association between c-*erb*B-2 amplification and disease recurrence (P=0.67).

The overall rate of proto-oncogene amplification (10%) in the primary breast carcinomas studied was lower than that commonly reported in the literature for two reasons. First, although most groups report an average of 25-30% gene amplification, the study populations usually include node-positive patients. Since the presence of positive axillary nodes has been shown to correlate with c-*erb*B-2 amplification[18], inclusion of this group of patients may increase the overall rate of gene amplification. Second, it is possible that a greater proportion of the primary tumors may be identified by assaying for c-*erb*B-2 at the m-RNA and protein levels. This possibility is supported by those studies which utilize various techniques (Southern, Northern and Western blots, immunohistochemistry) and show overexpression in the absence of gene amplification[22,25,26].

The major drawback of c-*erb*B-2 determination as a prognostic indicator was its low sensitivity as 85% of the recurring breast cancer patients harbored normal copy numbers of the proto-oncogene (Table 4.2). Approximately five of every six patients who are destined to relapse would go undetected by this test. Moreover, the use of c-*erb*B-2 elevation to assess patient prognosis had a low specificity. Two of the

eight patients were "false positives" inferring that 25% of the patients would receive adjuvant therapy with no benefit to them.

Another potential prognostic indicator of breast cancer is cellular DNA content. Studies of DNA ploidy in primary breast cancer have shown a significant association between the presence of aneuploid stem cell lines and decreased patient survival[27-34]. DNA aneuploidy has also been correlated to several clinico-pathological features of the tumor suggestive of poor clinical outcome[35-37].

In the current study, flow cytometric analysis revealed a range of DNA indices in the eight amplified patients. This suggests that primary breast cancer tumors overexpressing the c-*erb*B-2 proto-oncogene have variable amounts of DNA. Our results are in agreement with a recent study by Baak *et al.* in which increased HER-2/*neu* (synonymous with c-*erb*B-2) protein overexpression was found in 11% of diploid tumors[38]. However, the correlation between protein expression and ploidy remains controversial since Bacus *et al.* found that tumors overexpressing the HER-2/*neu* protein had a tetraploid or near-tetraploid DNA content[39].

The presence of an abnormal DNA content in 37 tumor blocks suggests an unfavorable prognosis in this subset of breast cancer patients. Twenty-two of these patients were "true positives" (eg. relapsed cases) who would benefit from additional therapy; however, the use of flow cytometric ploidy analysis as a predictive test is questionable as 41% of the patients who demonstrated abnormal DNA stemlines in their tumors remained disease-free. The specificity of this technique is also unreliable as a high proportion of patients who relapsed had a diploid DNA content in their tumor tissue. Furthermore, the incidence of DNA aneuploidy in the case and control groups was comparable, 42 vs 40% respectively. Since pairwise case-control matches were made to control for the potential confounding effects of various prognostic factors and other biologic variables, the use of DNA ploidy to accurately identify the subgroup of node-negative patients who will relapse is not

reliable. Hence, the administration of adjuvant therapy to a subset of patients demonstrating aneuploidy in their tumors is not sufficiently warranted.

Several investigators report that c-*erb*B-2 over-expression[18,26,40,41] and DNA aneuploidy[42-44] are predictors of relapse-free and overall survival in node-positive women; however, their prognostic significance in node-negative breast cancer remains controversial. From our study, combined c-*erb*B-2 amplification and DNA aneuploidy was an absolute predictor of poor prognosis in a limited number of patients. Only four of 78 patients who relapsed were accurately identified by the two prognostic indicators as being at an increased risk of recurrence. Furthermore, neither measurement alone could reliably identify those patients at greater risk of relapsing. As in the case group, individuals remaining disease-free during the same follow-up interval demonstrated proto-oncogene amplification and DNA aneuploidy in their tumor tissues. We conclude that the analysis of c-*erb*B-2 amplification and DNA aneuploidy offers limited clinical utility in node-negative breast cancer beyond that obtained with conventional prognostic guides.

REFERENCES

1. Haagensen CD. Treatment of curable carcinoma of the breast. Int J Radiat Oncol Biol Phsy 2:975-980, 1977

2. Fisher ER, Redmond C, Fisher B and other contributing National Surgical Adjuvant Breast and Bowel Project (NSABP) Investigators. Prognostic discriminants for 8-year survival for node-negative invasive breast cancer patients. Cancer 65:2121-2128, 1990

3. Valagussa P, Bonadonna G, Veronesi U. Patterns of relapse and survival following radical mastectomy: Analysis of 716 consecutive patients. Cancer 41:1170-1178, 1978

4. Fisher B, Redmond C, Dimitrov N and other contributing NSABP investigators. A randomized clinical trial evaluating sequential methotrexate and fluorouracil in the treatment of patients with node-negative breast cancer who have estrogen-receptor-negative tumors. N Engl J Med 320:473-478, 1989

5. Fisher B, Constantino J, Redmond C and other contributing NSABP investigators. A randomized clinical trial evaluating tamoxifen in the treatment of patients with node-negative breast cancer who have estrogen-receptor-positive tumors. N Engl J Med 320:479-484, 1989

6. Mansour EG, Gray R, Shatila AH *et al.* Efficacy of adjuvant chemotherapy in high-risk node-negative breast cancer. N Engl J Med 320:485-490, 1989

7. The Ludwig Breast Cancer Study Group. Prolonged disease-free survival after one course of perioperative adjuvant chemotherapy for node-negative breast cancer. N Engl J Med 320:491-496, 1989

8. Fourney RM, Dietrich KD, Paterson MC. Rapid DNA extraction and sensitive alkaline blotting protocol: Application for detection of gene rearrangement and amplification for clinical molecular diagnosis. Disease Markers 7:15-26, 1989

9. Paterson MC, Dietrich KD, Paterson AHG *et al.* Correlation between c-*erb*B-2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Res 51:556-567, 1991

10. Hedley DW, Friedlander ML, Taylor IW, Rugg C, Musgrove E. Method of analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 31:1333-1335, 1983

11. Babiak J, Poppema S. Automated procedure for dewaxing and rehydration of paraffin-embedded tissue sections for DNA flow cytometric analysis of breast tumors. Am J Clin Pathol 96:64-69, 1991

12. Hiddemann W, Schumann J, Andreeff M, *et al.* Convention on nomenclature for DNA cytometry. Cytometry 5:445-446, 1984

13. Harris JR, Hellman S, Canellos GP, Fisher B. Cancer of the Breast. In Cancer: Principles and Practice of Oncology. Vol 2 2nd Edition. Edited by VT Devita Jr, S Hellman, SA Rosenberg, J.B. Lippincott, Philadelphia, 1119-1177, 1985

14. Slamon DJ. Proto-oncogenes and human cancer. N Engl J Med 317:955-957, 1987

15. Schechter AL, Stern DF, Vaidyanathan L, *et al.* The *neu* oncogene: an *erb*-B-related gene encoding a 185,000M_r tumor antigen. Nature 312:513-516, 1984

16. Schechter AL, Hung MC, Vaidyanathan L, *et al.* The *neu* gene: an *erb*B-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science 229:976-978, 1985

17. Coussens L, Yang-Feng TL, Liao YC, *et al.* Tyrosine kinase receptor with extensive homology to EGR receptor shares chromosomal location with *neu* oncogene. Science 230:1132-1139, 1985

18. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human Breast Cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177-182, 1987

19. Ali IU, Campbell G, Lidereau R, Callahan R. Lack of evidence for the prognostic significance of c-*erb*B-2 amplification in human breast carcinoma. Oncogene Research 3:139-146, 1988

20. Zhou D-J, Ahuja H, Cline MJ. Proto-oncogene abnormalities in human breast cancer: c-*erb*B-2 amplification does not correlate with recurrence of disease. Oncogene 4:105-108, 1989

21. Wright C, Angus B, Nicholson S, *et al.* Expression of c-*erb*B-2 oncoprotein: A prognostic indicator in human breast cancer. Cancer Res 49:2087-2090, 1989

22. Berger MS, Locher GW, Saurer S, *et al.* Correlation of c-*erb*B-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. Cancer Res 48:1238-1243, 1988

23. Varley JM, Swallow JE, Brammer WJ, Whittaker JL, Walker RA. Alterations to either c-*erb*B-2 (*neu*) or c-*myc* proto-oncogenes in breast carcinoma correlate with poor short-term prognosis. Oncogene 1:423-430, 1987

24. Venter DJ, Tuzi NL, Kumar S, Gullick WJ. Overexpression of the c-*erb*B-2 oncoprotein in human breast carcinomas: immunohistological assessment correlates with gene amplification. Lancet ii:69-71, 1987

25. Guerin M, Barrois M, Terrier MJ, Spielmann M, Riou G. Overexpression of either c-*myc* or c-*erb*B-2/*neu* proto-oncogenes in human breast carcinoma: Correlation with poor prognosis. Oncogene Res 3:21-31, 1988

26. Tandon AK, Clark GM, Chamness GC, Ullrich A, McGuire WL. HER-2/*neu* oncogene protein and prognosis in breast cancer. J Clin Oncol 7:1120-1128, 1989

27. Atkin NB. Modal deoxyribonucleic acid value and survival in carinoma of the breast. Br Med J 1:271-272, 1972

28. Atkin NB, Kay R. Prognostic significance of modal DNA value and other factors in malignant tumors, based on 1465 cases. Br J Cancer 40:210-221, 1979

29. Auer G, Eriksson E, Azavedo E, Caspersson T, Wallgren A. Prognostic significance of nuclear DNA content in mammary adenocarcinomas in humans. Cancer Res 44:394-396, 1984

30. Hedley DW, Friedlander ML, Taylor IW. Application of DNA flow cytometry to paraffin-embedded archival material for the study of aneuploidy and its clinical significance. Cytometry 6:327-333, 1985

31. Coulson PB, Thornthwaite JT, Woolley TW, Sugarbaker EV, Seckinger D. Prognostic indicators including DNA histogram type, receptor content, and staging related to human breast cancer patient survival. Cancer Res 44:4187-4196, 1984

32. Kallioniemi O-P, Heitanen T, Mattila J, Lehtinen M, Lauslahti K, Koivula T. Aneuploid DNA content and high S-phase fraction of tumor cells are related to poor prognosis in patients with primary breast cancer. Eur J Cancer Clin Oncol 23;277-282, 1987

33. Clark GM, Dressler LG, Owens MA, Pounds G, Oldaker T, McGuire WL. Prediction of relapse or survival in patients with node-negative breast cancer by DNA flow cytometry. N Engl J Med 320:627-633, 1989

34. Hedley DW, Rugg CA, Ng AB, Taylor IW. Influence of cellular DNA content of disease free survival of stage II breast cancer patients. Cancer Res 44:5395-5398, 1984

35. Thorud E, Fossa SD, Vaage S, *et al.* Primary breast cancer. Flow cytometric DNA pattern in relation to clinical and histopathologic characteristics. Cancer 57:808-811, 1986

36. Olszewski W, Darzynkiewicz Z, Rosen PP, Schwartz MK, Melamed M. Flow cytometry of breast carcinoma: I. Relation of DNA ploidy level to histology and estrogen receptor. Cancer 48:980-984, 1981

37. Moran RE, Black MM, Alpert L, Straus MJ. Correlation of cell-cycle kinetics, hormone receptors, histopathology and nodal status in human breast cancer. Cancer 54:1586-1590, 1984

38. Baak JP, Chin D, van Diest PJ, Ortiz R, Matze-Cok P, Bacus SS. Comparative long-term prognostic value of quantitative HER-2/*neu* protein expression, DNA ploidy and morphometric and clinical features in paraffin-embedded invasive breast cancer. Lab Investigation 64:215-223, 1991

39. Bacus SS, Bacus JW, Slamon DJ, Press MF. HER-2/*neu* oncogene expression and DNA ploidy analysis in breast cancer. Arch Pathol Lab Med 114:164-169, 1990

40. Slamon DJ, Godolphin W, Jones LA, *et al.* Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science 244:707-712, 1989

41. Tsuda H, Hirohashi S, Shimosato Y, *et al*. Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: *hst*-1/*int*-2 and c-*erb*B-2/*ear*-1. Cancer Res 49:3104-3108, 1989

42. Hedley DW, Rugg CA, Gelber RD. Associaton of DNA index and S-phase fraction with prognosis of nodes positive early breast cancer. Cancer Res 47:4729-4735, 1987

43. Cornelisse CJ, van de Velde CJ, Caspers RJ, Moolenaar AJ, Hermans J. DNA ploidy and survival in breast cancer patients. Cytometry 8:225-234, 1987

44. Klintenberg C, Wallgren A, Bjelkenkrantz K, *et al*. DNA distribution, cytosol estrogen receptors and axillary nodes as prognostic predictors in breast carcinoma. Acta Radiol Oncol 24:253-258, 1985

CHAPTER FIVE

INHERENT RADIOSENSITIVITY TESTING OF TUMOR BIOPSIES OBTAINED FROM PATIENTS WITH CARCINOMA OF THE CERVIX OR ENDOMETRIUM*

5.1 INTRODUCTION

When carcinoma of the cervix is confined to the pelvis it is potentially curable by radiotherapy[1]. The overall prognosis depends on a number of clinical factors but stage, tumor bulk and the presence of pelvic lymph node metastases are the most important. Uncontrolled pelvic disease is a significant cause of mortality and morbitity in patients where primary radiotherapy is not curative[2]. One of the challenges facing oncologists is the need to identify the biological and/or molecular factors which characterize this subgroup of patients at risk for treatment failure and to design appropriate alternative therapies for them. Because many patients with gynecological malignancies are treated with radiotherapy either as a single modality or in combination with surgery or chemotherapy, there is considerable interest in examining the role that tumor radiosensitivity may play in determining treatment response. When compared to factors such as tumor clonogen doubling time, hypoxic fraction or tumor clonogen number, inherent radiosensitivity is theoretically the most likely to correlate with radiation treatment response[3]. The correspondence between inherent radiosensitivity measured *in-vitro* and the *in-vivo* sensitivity of tumors to a fractionated course of radiation has recently been demonstrated in animal tumor models[4]. The clinical relevance of such assays has yet to be conclusively established. However, work by Brock and colleagues[5] measuring the inherent radiosensitivity of biopsy specimens obtained from a series of patients with head and neck is encouraging. Their initial results indicate that tumor biopsies

* A version of this chapter has been accepted: Allalunis-Turner MJ, Pearcy RG, Barron GM, Buryn DA, Babiak JC, Honore LH. Inherent Radiosensitivity Testing of Tumor Biopsies Obtained from Patients with Carcinoma of the Cervix or Endometrium. Radiotherapy Oncology, September, 1991

obtained from patients who subsequently developed a local recurrence were, on average, more radioresistant than were those of patients who achieved local control.

If it can be demonstrated that the inherent radiosensitivity of human tumor biopsies has correlative value with the clinical response to treatment with radiation, then assays which reliably and accurately measure this parameter could have significant impact on the design of individualized cancer therapy. For example, in the case of gynecological tumors, stage 1B cervical cancer may be treated by either radiotherapy or surgery. While a number of factors are important in determining the best treatment for each patient, a patient with a reliably predicted resistant cancer would be best treated surgically. Similarly, patients with advanced cervix cancer might be better treated by combined radiotherapy and chemotherapy and this could be tested in future clinical trials.

At the present time, several laboratories are testing and developing *in-vitro* assay systems for measuring inherent tumor radiosensitivity. In this chapter, we report the results of our studies of the inherent radiosensitivity of tumor biopsies obtained from patients with carcinoma of the uterine cervix or endometrium and compare these estimates with the DNA index of the tumor specimens and with histological type and grade.

5.2 MATERIALS AND METHODS

5.2.1 Biopsies

A portion of the biopsy taken for confirmation of histological subtype in patients with carcinoma of the uterine cervix or endometrium was used in these studies. All procedures were approved in advance by the Institute's Ethics Committee on Human Expe.imentation. Biopsies were minced with a scalpel and then dissociated for 30 minutes at 37^{o}C in an enzyme cocktail consisting of DNAse (0.04%), Pronase (0.05%) and collagenase (0.025%). The resulting cell suspension

was passed through a wire mesh screen to remove clumps and debris, washed twice and counted. An aliquot of cells was seeded onto 60 mm tissue culture dishes in complete medium [F12/DMEM (DULBECCO'S MODIFIED EAGLE'S MEDIA) containing 20% fetal calf serum and 1 mM HEPES buffer]. The remaining cells were washed three times in PBS, fixed in 70% ethanol and stored for flow cytometric analysis.

5.2.2 Inherent Radiosensitivity

Early passage (3-5) cell lines were trypsinized when nearly confluent and irradiated in suspension at 37°C. A mixture of 5% CO_2 in air was flowed through the cell suspension chamber at approximately 1 L/min for 15 minutes prior to and during the irradiation procedure. A ^{137}Cs source operating at a dose rate of 1.86 Gy per minute was used to deliver doses of 1 to 2 Gy to the cells. Aliquots of cells were removed following each dose of radiation and were held on ice prior to plating. Total doses ranged from 1 to 10 Gy. Known numbers of cells were plated onto 60 mm tissue culture dishes into complete medium. Nine to 15 plates were used per dose point and were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. At weekly intervals, spent medium was removed and replaced with fresh medium and plates were returned to the incubator. Three weeks after irradiation, the plates were stained with methylene blue and colonies greater than 50 cells were counted by eye. The surviving fraction of cells was determined and the data were fit to the linear quadratic model of cell inactivation using a computer based program. The surviving fraction at 2 Gy was determined by inspection of the survival curve.

5.2.3 Flow Cytometry

Cells were prepared for flow cytometric DNA analysis according to techniques previously described[6]. Normal human lymphocytes served as a diploid

control and were prepared as were the tumor cells. Cells were analyzed using an EPICS V® or FACScan® flow cytometer equipped with a 488 nm argon ion laser for excitation. For this study, diploid tumor cell samples contained a single G_0/G_1 peak with a mean channel number being the same as that of the diploid reference population. DNA aneuploid samples were those in which a G_0/G_1 peak separate from that of the diploid reference peak was identified. Hyperdiploid tumors were identified by broad shoulders on the G_0/G_1 reference peaks and as distinct second populations on the dot plot analyses.

5.3 RESULTS

The diagnosis, clinical stage and age of the patients from whom biopsy specimen were obtained is listed in Table 5.1. The SF2 values, plating efficiencies and DNA ploidy determinations are also shown. The SF2 values and plating efficiencies are average values derived from two or more separate experiments.

The relative cumulative frequency distributions of SF2 values for the cervical and endometrial carcinomas are shown in Figures 5.1 and 5.2. The mean (± SD) SF2 value for 26 cervical carcinomas was 0.29 ± 0.12 (range = 0.11 - 0.59); for 18 endometrial carcinomas, 0.30 ± 0.15 (range = 0.11 - 0.67).

In Figure 5.3, the SF2 values determined for both cervical and endometrial carcinomas were plotted as a function of DNA ploidy of the original biopsy specimen. No correlation between inherent tumor cell radiosensitivity and DNA ploidy was evident.

In Figure 5.4, the average SF2 values of the tumors were plotted as a function of the *in-vitro* plating efficiency. No significant correlation was observed. In addition, no correlation was observed between the SF2 values and the histological type or grade of the tumors (data not shown).

Table 5.1. Summary of Patient and Tumor Data. The diagnosis, clinical stage of the tumor and age of the patients from whom the biopsies were obtained are provoded. The tumor inherent radiosensitivity (SF2), DNA ploidy and plating efficiency (PE) are also shown. The SF2 and PE values are average values derived from two or more separate experiments.

Cervical Tumors

Patient #	Age	Pathology	Stage	Ploidy	PE	SF2
A. Squamous Cell Carcinoma						
HT54	48	Gr II	3B	Diploid	.08	.22
HT61	32	Gr III	1B	Diploid	.23	.41
HT62	47	Gr II	1B	Aneuploid	.11	.20
HT67	41	Gr II/III	3B	Aneuploid	.63	.32
HT81	41	Gr III	1B	Diploid	.06	.26
HT100	28	Gr III	2B	Diploid	.03	.45
HT102	44	Gr II	3B	Diploid	.11	.13
HT103	45	Gr III	2B	Diploid	.20	.25
HT121	44	Gr III	1B	Diploid	.01	.19
HT131	26	Gr III	1B	Diploid	.09	.24
HT133	37	Gr III	1B	Aneuploid	.05	.30
HT134	70	Gr III	4A	Aneuploid	.08	.20
HT138	50	Gr III	2B	Aneuploid	.01	.22
HT141	41	Gr III	2B	Aneuploid	.03	.31
HT151	56	Gr II	2B	Diploid	.04	.40

Patient #	Age	Pathology	Stage	Ploidy	PE	SF2
HT152	45	Gr III Small Cell	1B	Diploid	.09	.17
HT153	44	Small and Large Cell	1B	Aneuploid	.01	.39
HT171	32	Gr III	1B	Aneuploid	.08	.33
B. Adenocarcinoma						
HT 77	57	Gr III	4	Diploid	.23	.28
HT129	36	Gr I	1B	Aneuploid	.13	.59
HT137	36	Gr I	1B	Aneuploid	.07	.38
HT140	37	Gr I	1B	Aneuploid	.07	.14
HT172	49	Gr III	1B	Diploid	.01	.20
C. Glassy Cell Carcinoma						
HT93	35	Gr III	1B	Diploid	.18	.41
HT116	26	Gr III	2B	Aneuploid	.07	.33
D. Other (Mixed Mullerian)						
HT69	64	-	4	Aneuploid	.28	.17

Endometrial Tumors

Patient #	Age	Pathology	Stage	Ploidy	PE	SF2
A. Adenocarcinoma						
HT42	63	Gr I	1A	Diploid	.12	.31
HT56	57	Gr I	1B	Hyper-diploid	.15	.24
HT74	60	Gr I	1	Diploid	.08	.22
HT80	71	Gr I	1A	Hyper-diploid	.32	.42
HT112	71	Gr I	1A	Diploid	.04	.37
HT136	50	Gr I	1B	Diploid	.15	.21
HT157	53	Gr I	1B	Diploid	.07	.45
HT75	63	Gr II	1B	Diploid	.19	.16
HT82	68	Gr II	1A	Diploid	.23	.17
HT118	66	Gr II	1B	Diploid	.06	.25
HT123	63	Gr II	1B	Aneuploid	.07	.43
HT158	58	Gr II	1A	Diploid	.01	.22
HT65	53	Gr III	2	Not Avail-able	.40	.18
HT106	72	Gr III	2	Aneuploid	.02	.67
HT107	60	Gr III	1-2	Aneuploid	.05	.29
HT124	84	Gr III	2B	Diploid	.02	.11
HT155	32	Gr III	2	Aneuploid	.04	.51

Patient #	Age	Pathology	Stage	Ploidy	PE	SE2
B. Serous Papillary						
HT52	67	-	1B	Aneuploid	.29	.11

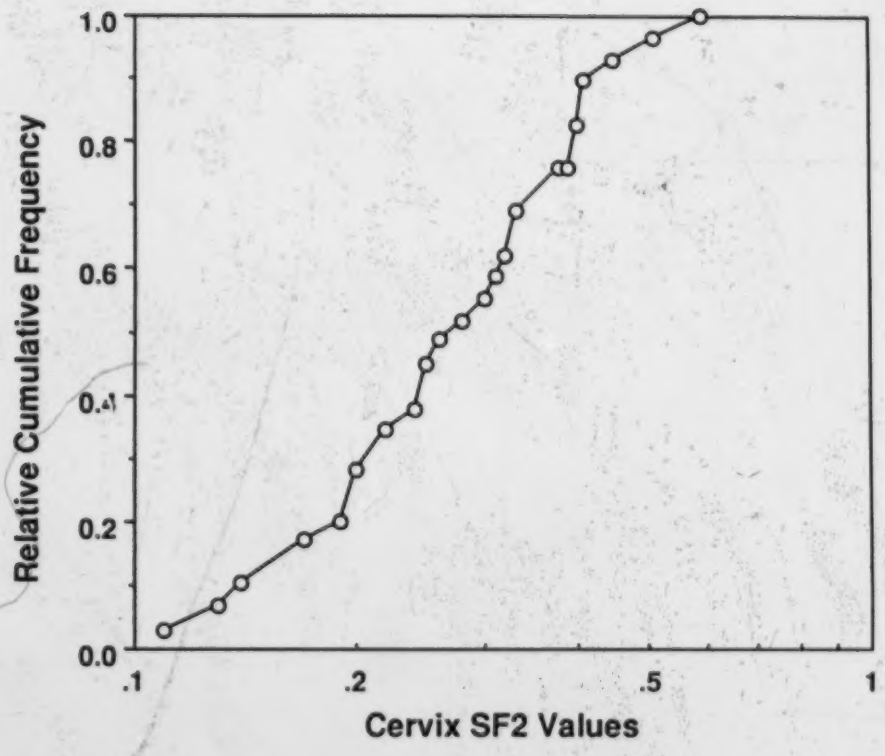


FIGURE 5.1

The relative cumulative frequency distribution of the SF2 values of tumor biopsies obtained from patients with carcinoma of the cervix.

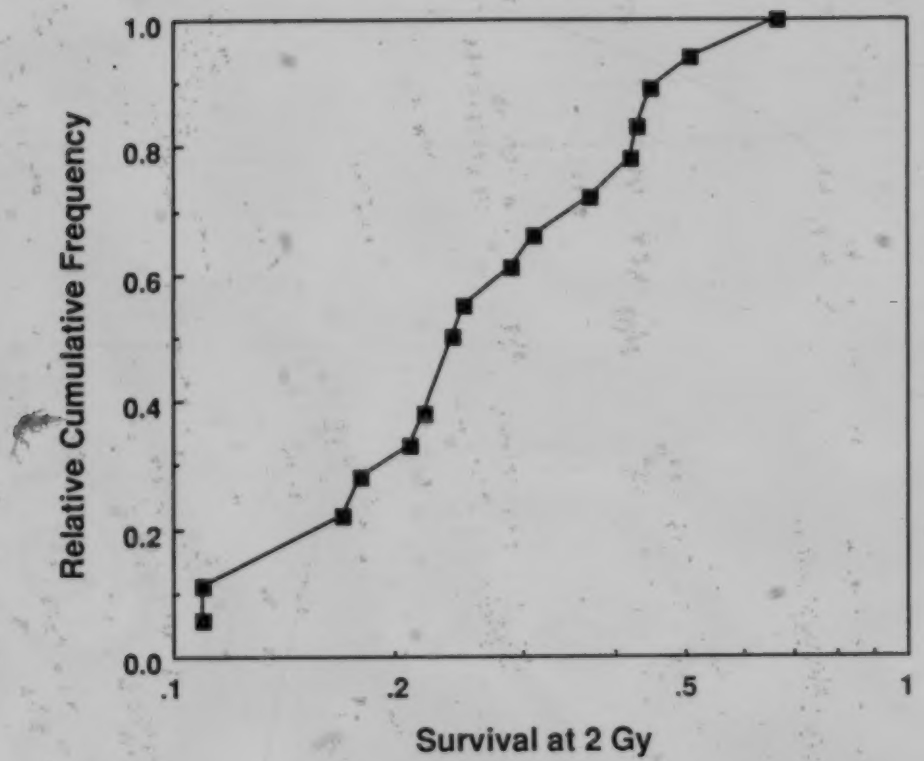


FIGURE 5.2

The relative cumulative frequency distribution of the SF2 values of tumor biopsies obtained from patients with carcinoma of the endometrium.

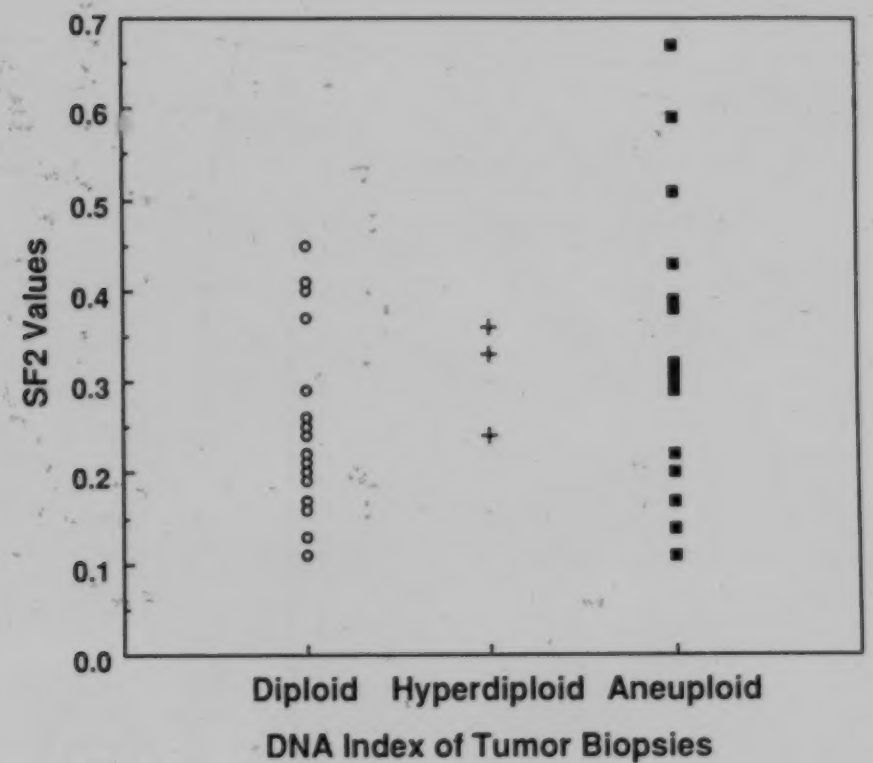


FIGURE 5.3

The distribution of SF2 values among diploid, hyperdiploid or aneuploid tumors of the cervix and endometrium.

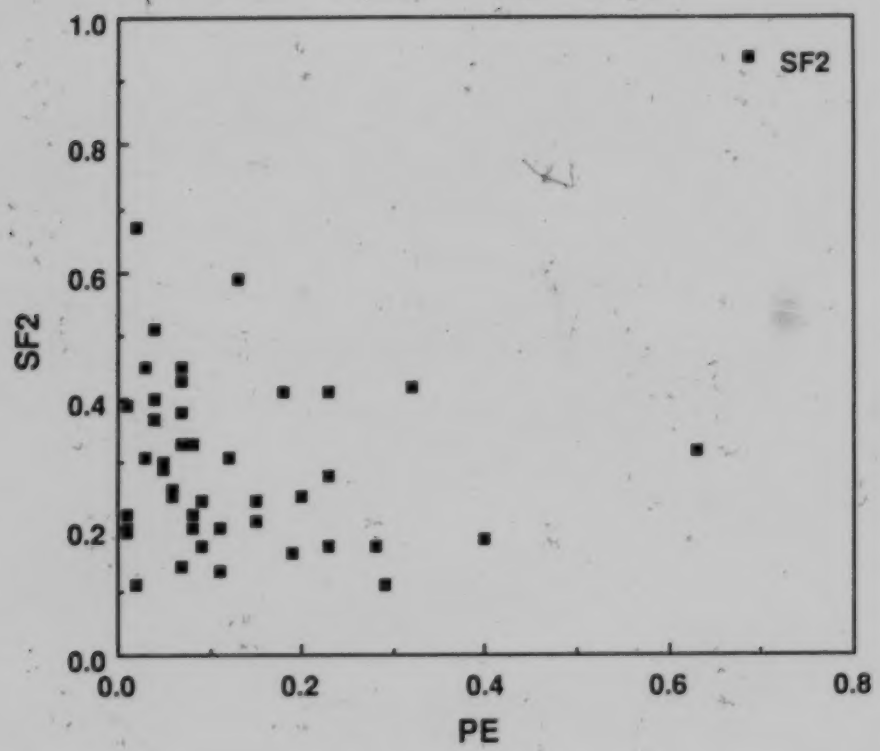


FIGURE 5.4

SF2 values for cervical and endometrial tumors plotted as a function of the in vitro plating efficiency.

5.4 DISCUSSION

Cervical cancer is curable by radiotherapy which is the mainstay of treatment for this condition[1]. Endometrial cancer is also curable by radiotherapy alone[7] although in most cases radiotherapy is used in combination with surgery. The results of our study which measured the inherent radiosensitivity of tumor biopsies taken prior to therapy are in agreement with that clinical observation. For cervical or endometrial carcinomas, the mean (± SD) SF2 values were 0.29 ± 0.12 and 0.30 ± 0.13 respectively. The mean SF2 value for the 26 cervical tumors in this study is comparable to those previously reported for this tumor type. Using the Courtnay assay and fresh biopsy material from 46 tumors, West and colleagues reported a mean (± SEM) SF2 value of 0.45 ± 0.03 (range = 0.14 - 0.97)[8]. In their study of 5 biopsy-derived tumor cell lines grown in monolayer cultures, Kelland and Steele reported SF2 values of 0.23, 0.33, 0.45, 0.56 and 0.60[9]. Because of differences in experimental procedures and assay systems which exist among laboratories, and the heterogeneity which exists among tumors, it is unlikely that a unique value for the radiosensitivity of a given histological class of tumor will ever be defined. However, the fact that these three separate studies of cervical carcinoma have all resulted in mean SF2 values which are characteristic of moderately radiosensitive tumors is encouraging. Another feature in common to the three studies of cervical carcinoma is the broad range of SF2 values. In order for such assays to be clinically useful, they must be able to detect significant differences between tumors in order that patients with unusually sensitive or resistant tumors can be identified[10]. The results presented in this study suggest that discrimination between sensitive and resistant tumors may be possible.

Little comparative information is available concerning the inherent radiosensitivity of endometrial carcinomas. Like cervical carcinoma, endometrial carcinoma is classed as being moderately radioresponsive. The SF2 values derived

in our study are in agreement with this clinical observation. As well, the broad range of SF2 values noted for the cervical carcinomas was also observed for the endometrial carcinomas.

In the previously reported studies of cervical carcinoma, no correlation between the plating efficiency and the degree of radiosensitivity was apparent[8]. This was also the case for our own studies with tumors of the cervix and endometrium and suggests that the radiosensitivity of tumors is not a function of their ability to adapt well to growth *in-vitro*. As well, no correlation was apparent between the inherent radiosensitivity of the tumors and the histological type or the tumor grade. Although only 5 cervical adenocarcinomas were included in our study, they were not on average more radioresistant than were squamous cell carcinomas. This finding is consistent with the opinion of Fletcher that stage for stage, cervical glandular and squamous cell carcinomas are similarly responsive to radiotherapy[11].

In order for predictive assays to be of use to the clinician, they must provide results which are accurate, highly reproducible and available within a reasonably short period of time. The early passage human tumor cell lines and the clonogenic assay system used to generate these data, while useful for experimental studies, have several drawbacks which prevent their use on a routine basis for analysis of clinical material. For example, the success rate for establishing the cell lines used in these studies was approximately 40%. In addition, several weeks to months were often required for a complete evaluation of the results. For these reasons, we investigated the possibility that determination of tumor DNA ploidy might be useful as a rapid screening system for identifying radioresistant or radiosensitive tumor samples.

Studies by Dyson *et al.* have suggested that aneuploid cervical carcinomas are more radioresponsive than are diploid carcinomas, and, in patients with late stage disease, have a lower incidence of local recurrence[12]. Rutgers *et al.* also reported that among women with well-to-moderately differentiated carcinomas of the cervix

treated exclusively with radiotherapy, tumor aneuploidy conferred a significantly better prognosis[13]. Although these clinical studies suggest that aneuploid cervical carcinomas may be inherently more radiosensitive, a direct comparison of the radiosensitivity of tumor biopsies obtained from individual patients and the DNA index of the same biopsy specimen has previously not been available. In this study, the DNA index of a series of biopsy specimens obtained from patients with carcinoma of the cervix and endometrium was compared to the SF2 values determined for the corresponding tumor cell lines. As shown in Figure 5.3 for both cervical and endometrial carcinomas, there is no correlation between inherent radiosensitivity of the tumor cell line and the DNA index of the tumor biopsy. Because of concern that *in-vitro* passage of the tumor cell lines may have resulted in a DNA ploidy which differed from that of the original biopsy specimen, these data were reanalyzed by using the cell line DNA indices and comparing these to the SF2 values. No correlation between DNA ploidy and radiosensitivity was observed, suggesting that lack of correlation between tumor SF2 values and the original biospy DNA index was not an *in-vitro* artifact. These results also suggest that flow cytometric analysis of tumor biopsy DNA index alone will not have predictive value as regards to tumor cell radiosensitivity.

A problem inherent with the use of biopsy material in any predictive assay is that of sampling error. The biopsy perforce is obtained from the surface of the tumor and may not be representative of the tumor as a whole or of the inaccessible regions of a deeply infiltrating tumor. In the present study, a portion of the biopsy used for SF2 testing and DNA analysis was submitted for pathological evaluation. In all cases, the tumor material used for these predictive assays was judged to be similar in type and grade to other parts of the same tumor obtained on previous occasions. While similarity in pathology cannot guarantee that uniformity in

inherent radiosensitivity or DNA index exists within the tumor, it does suggest that the biopsies used in these studies were not grossly aberrant.

The validity of using *in-vitro* assays to predict response to radiotherapy will ultimately depend on the ability to correlate such measurements with the clinical response of each patient tested. Such an evaluation is in progress for the patients described in this study.

REFERENCES

1. Fletcher GH. Cancer of the uterine cervix. Janeway Lecture 1970. Amer J Roentgen Rad Ther Nucl Med 3:225-242, 1970

2. Brady LW, Markow AM, DeEulis T, Lewis Jr GC. Treatment of advanced and recurrent gynecologic cancer. Cancer 60:2081-2093, 1987

3. Tucker SL, Thames HD. The effect of patient-to-patient variability on the accuracy of predictive assays of tumor response to radiotherapy: as theoretical evaluation. Int J Radiat Oncol Biol Phys 17:147-157, 1989

4. Bristow RG, Hill RP. Comparison between *in-vitro* radiosensitivity and *in-vivo* response in murine tumor cell lines II: Radioresponse following fractionated treatment and *in-vitro/in-vivo* correlations. Int J Radiat Oncol Biol Phys 18:1331-1345, 1990

5. Brock WA, Baker FL, Wike JL, Sivon SL, Peters LJ. Cellular radiosensitivity of head and neck squamous cell carcinomas and local tumor control. Int J Radiat Oncol Biol Phys 18:1283-1286, 1990

6. Clevenger CV, Bauer KD, Epstein AL. A method for simultaneous nuclear immunofluorescence and DNA content quantitation using monoclonal antibodies and flow cytometry. Cytometry 6:208-214, 1985

7. Langdon RC, Fletcher GH, Delclos L, Whorton JT. Irradiation of endometrial cancer in patients with medical contraindication to surgery or with unresectable lesions. Amer J Roentgenol Rad Ther Nucl Med 126:148-154, 1976

8. West CML, Davidson SE, Hunter RD. Evaluation of surviving fraction at 2 Gy as a potential prognostic factor for the radiotherapy of carcinoma of the cervix. Int J Radiat Biol 56:761-765, 1989

9. Kelland LR, Steel GG. Differences in radiation response among human cervix carcinoma cell lines. Radiother Oncol 13:225-232, 1988

10. Peters LJ. Inherent radiosensitivity of tumor and normal tissue cells as a predictor of human tumor response. Radiother Oncol 17:177-190, 1990

11. Fletcher GH. In Textbook of Radiotherapy, pp 667, Lea and Febiger, Philadelphia, 1973

12. Dyson JED, Joslin CAF, Quirke P, Rothwell RI, Bird CC. Quantitation by flow cytofluormetry of response of tumors of the uterine cervix to radiotherapy. Br J Radiol 58:41-50, 1985

13. Rutgers DH, van der Linden PM, van Peperzeel HA. DNA-flow cytometry of squamous cell carcinomas from the human uterine cervix: the identification of prognostically different subgroups. Radiother Oncol 7:249-258, 1986

CHAPTER SIX

GENERAL DISCUSSION

The principle subject explored by this thesis was the analysis of cellular DNA content by flow cytometric analysis. This technology marks the beginning of a new era of science with unlimited potential. The evolution of Hedley's technique to perform flow cytometric DNA analysis on paraffin-embedded tumors has benefitted studies relating prognosis to DNA content abnormalities. However, there is little consensus on the technique. There is considerable variation in the methodology of sample preparation, staining and data interpretation. Moreover, a considerable amount of time and labor is required to complete the preparation of paraffin samples, which often yield inferior results. The work reported in this thesis has identified several sources of variation and addressed some of the difficulties of processing archival material for flow cytometric DNA analysis. The automation of a significant portion of the routine processing required for paraffin-embedded tissue, as detailed in Chapter 3, is one step closer to improving the efficiency of this method. The second focus of this thesis was to investigate the clinical utility of flow cytometric DNA measurements. The data presented in Chapters 4 and 5 examined the nature of DNA content abnormalities and their relation to prognosis in solid tumors of breast and cervix.

6.1 ADDRESSING THE TECHNICAL LIMITATIONS

6.1.1 Increased Coefficients of Variation

Increased coefficients of variation are an inherent feature of DNA histograms from paraffin-embedded tissue samples. Broad G_0/G_1 peaks with high CVs may obscure interpretation of the data. To overcome this, a maximum CV of 6.0 was assigned as the upper limit for acceptance of a DNA histogram. When CVs

exceeded this value and a second G_0/G_1 peak could not be identified, the samples were reanalyzed using an additional tumor block if available. A subsequent CV of 6.0 or greater was considered an "uninterpretable histogram" and the tumor was omitted from inclusion in the study. Using this criterion, the possibility of overlooking stemlines with a "near-diploid" DNA content that may be evident in fresh tissue specimens was eliminated.

6.1.2 The Fixation Process

Fixation is a valuable process for the short- or long-term preservation of biological samples. In many instances, however, the significance of the type of fixative and duration of the process on the subsequent quality of DNA histograms is overlooked. First, fixatives fall into two broad categories: those, such as the aldehydes, that are additive and result in the generation of chemical bonds between molecules, and those, such as the alcohols, that are non-additive and are lipid solubilizing. An evaluation by Hedley of the effect of several fixatives on DNA histogram quality confirmed that neutral formalin yielded the lowest CVs[1]. Thus, only paraffin blocks originally fixed in formalin were considered for this study. Second, it is known that fixative penetration is variable, therefore it is reasonable to assume that cells in the central mass of tissue, particularly in large pieces, may not be fixed to the same extent as cells on the peripheral edge. A DNA fluorochrome would thus present variations in its ability to penetrate individual cells. To avoid this variation, small tumor specimens (1cm x 1cm) were selected for flow cytometric analysis and complete tissue fixation was confirmed by microscopic observation of a thin section stained with hematoxylin and eosin.

6.1.3 Tissue Cellularity

When a paraffin block is dedicated to flow cytometry, it is important to ensure that the block contains cells of diagnostic relevance or is representative of the tumor. To verify the actual cellular content of the paraffin block, a conventional histological section adjacent to the thick sections disaggregated for flow cytometric DNA analysis was examined. In cases where insufficient proportions of normal and tumor cells were present, a second tumor block was sampled. Furthermore, the amount of debris generated by the analysis of paraffin-embedded material was minimized by excluding tumors with hemorrhagic areas and evidence of necrotic tissue.

6.1.4 Section Thickness

The procedural step of greatest significance affecting the final quality of DNA histograms is sectioning of the tumor block. The effect of section thickness was systemically studied by Stephenson *et al.* who concluded that sections measuring 50-μm were optimal for flow cytometric DNA analysis[2]. They found this thickness resulted in decreased baseline nuclear debris and enhanced recovery of DNA aneuploid populations. Given the advantages of 50-μm sections, this thickness was used for preparing all paraffin samples in this study.

6.1.5 Enzymatic Digestion

The enzyme digestion step, while necessary to produce a monodisperse suspension of nuclei from the rehydrated tissue sections, may also lead to increasing amounts of debris. Prolonged incubation causes high baseline debris presumably because of increasing DNA fragmentation. To limit this, tissue sections were incubated for the recommended 30 minutes according to Hedley's original procedure for processing paraffin-embedded material for flow cytometric DNA

analysis[3]. To further enhance the recovery of intact nuclei, tissue sections were mechanically disrupted with brief vortexing at frequent intervals during the enzyme incubation. By adding this mechanical disaggregation step to Hedley's original method, the resulting DNA histograms were of better quality (lower CVs) and the recovery of nuclear populations was enhanced.

6.1.6 Lack of Reliable Standards

The analysis of paraffin-embedded material is not entirely suited for flow cytometric analysis because of the lack of reliable standards to determine DNA ploidy. While this limitation is unsurmountable at present, it was partially circumvented in this study by using paraffin blocks containing malignant and non-malignant cells, thereby allowing the normal host cells to mark the diploid reference point. When this was unavailable, a separate block of non-involved lymph node from the same patient, fixed and processed in an identical manner, was chosen as the diploid standard. Moreover, in the analysis of DNA histograms, two or more discrete G_0/G_1 peaks had to be present to support an interpretation of DNA aneuploidy. Since the convention for determining DI from paraffin blocks is to assume that the G_0/G_1 peak with the lowest DNA content is diploid[4], the limitation that hypodiploid tumors can not be identified remains a reality despite efforts to overcome it. This not only affects the percentage of aneuploid tumors reported in the literature, but overlooks the important facet of malignancy that deletion of genetic material is increasingly being recognized as an important cause of malignant progression[5]. If the true significance of cellular DNA content is to be determined, a more accurate means of measuring DNA content aberration in paraffin-embedded tissue samples must be found.

6.2 SAMPLE PREPARATION

6.2.1 Automated Dewaxing action and Rehydration Procedure

The most labour-intensive step of preparing paraffin-embedded material for flow cytometric DNA analysis is dewaxing and rehydrating the tissue. As detailed in Chapter 3, an automated procedure was developed to complete this task. This refinement in methodology offers significant advantages over the manual procedure. Most important, the efficiency of processing paraffin-embedded samples is improved by the three-fold increase of samples that can be batched during a single run and by the enhanced productivity of the technologist. Second, the dewaxing and rehydration of tissue while enclosed in a cassette minimized the chance of losing cellular populations. In the existing manual method, serial aspirations mechanically disrupt the integrity of the tissue. Smaller fragments of tissue that do not pellet with centrifugation are easily aspirated and discarded with each change in solution. This results not only in a decrease in cell recovery, but also in nuclear suspensions not being entirely representative of the original tumor sampled. Third, by using an instrument equipped with an automatic timer, the samples were consistently dewaxed and rehydrated for equivalent time intervals. In addition, the processor was capable of rotating the cassette carrier from side-to-side to ensure uniform mixing throughout the procedure. This is in contrast to the manual method in which tissue sections are left idle in the test tube. As forementioned, mixing is not recommended with the manual method as it dislodges fragments of tissue that may be discarded. Moreover, the possibility exists that tissue sections are not transferred through the solutions at exact time intervals when this step is performed manually. The demonstration of an automated procedure for dewaxing and rehydrating paraffin-embedded tissue makes flow cytometric DNA analysis a more practical procedure in the laboratory by reducing the labour involved in sample preparation.

6.2.2 Standardized DNA Assay Procedure

Another significant limitation associated with the paraffin technique is the lack of standardized assay procedures. With the increasing popularity of DNA flow cytometry for clinical applications, there is a critical need for standardization in DNA sample preparation and staining technology. Although not reported in this thesis, a commercially available assay system, the Coulter DNA-Prep, was evaluated. The DNA-Prep is an automated sample preparation workstation complete with ready-to-use reagents that prepares intact cells or isolated nuclei for reproducible lysis, permeabilizing and staining for the consistent, quantitative measurement of cellular DNA content by flow cytometry. The DNA-Prep reagent system eliminates frequent preparation and special quality control handling of propidium iodide solutions. The reagents also require no reconstitution, dilution or centrifugation protecting the technologist from exposure to a potentially harmful chemical. The interlaboratory variation in sample preparation and staining is also minimized using commercially available, quality-tested reagents. The concentration of propidium iodide and digestion activity of RNAse are consistent from lot-to-lot providing consistent DNA histograms. The assay system is also equipped with an internal standard of consistent DNA content to monitor instrument linearity, resolution and CVs. The ability to set a constant reference point provides a consistent ratio of fluorescence to the sample's diploid peak, and thus, a reliable standard.

The evaluation of this standardized assay system included a five day trial of staining the same paraffin-embedded tumor sample using two methods: the technically improved procedure outlined in Chapter 3 and the DNA-Prep system. While the DNA staining patterns and CVs were comparable in both methods, DNA-Prep was superior to the current technique in recovering rare aneuploid populations. The total nuclear counts were also higher with the automated staining

system suggesting that little or no loss of nuclei had occurred during the staining process. The use of the DNA-Prep system also eliminated the variables and labor inherent in the manual technique of sample preparation and staining.

The automation of processing paraffin-embedded material for flow cytometric DNA analysis is a crucial step toward formulating standard methodologies and reducing the disadvantages of Hedley's original paraffin procedure. The automated procedure for dewaxing and rehydrating tissue samples eliminates tedious manual procedures and standardizes a portion of the technique. In addition, the DNA-Prep reduces the variation in sample preparation and DNA staining among different laboratories. Both these advances are a step closer to devising a single standardized method of preparing paraffin-embedded archival material for DNA flow cytometry. Using this approach, results from several laboratories may be compared to provide conclusive evidence regarding the significance of cellular DNA content.

6.3 LACK OF CONSENSUS IN DATA INTERPRETATION

There is broad agreement on how the results of DNA analysis should be displayed and reported, however, there are varying opinions of the definition of DNA aneuploidy[6]. To facilitate the understanding of data and to standardize the terminology for DNA analysis among laboratories, the Society for Analytical Cytology has provided guidelines for expressing abnormalities of DNA content[6]. These guidelines cover: staining of DNA, cytogenetic and cytometric terminology, DNA index determination, resolution of measurements and cytometric standards. With regards to the definition of DNA aneuploidy, the committee states that "the diagnosis of DNA aneuploidy should be reported only when at least two separate G_0/G_1 peaks are demonstrated". This definition of DNA aneuploidy was applied to the analysis of each DNA histogram generated by this work. It was particularly

useful in the analysis of paraffin-embedded tumors where the absence of reliable standards preclude the accurate determination of DNA index. The lack of consensus on histogram classification is another area of considerable variation in data interpretation. This variation arises from the subjective manner in which DNA histograms are evaluated. To interpret the data in this study accurately and objectively, a calibration constant (See Appendix), determined by PI-stained calf thymocytes, was initially established prior to the analysis of tumor samples. This constant is based on the mathematical assumption that the fluorescence intensity of cells in each compartment of the cell cycle was normally distributed, with the mean of the G_2M (tetraploid) compartments having a channel location approximately twice that of the G_0/G_1 (diploid) compartment. This calibration constant, calculated to be 1.97, helped determine the position of tetraploid populations and clarified near-tetraploid aneuploid peaks. The presence of tetraploidy was also considered different from DNA aneuploid since some normal tissues (liver) exhibit tetraploid cells with mononuclear or binuclear morphology[7]. In this investigation of breast, cervical and endometrial tumors, the tetraploid populations had to comprise at least 15% of the total number of cells and demonstrate a corresponding G_2M (8N) peak to be considered DNA aneuploid. In summary, all DNA histograms generated by this work were interpreted and classified objectively using criteria specific to DNA flow cytometry.

6.4 CLINICAL UTILITY OF DNA FLOW CYTOMETRY

The potential applications of flow cytometric DNA determinations were greatly extended by the introduction of Hedley's technique for the analysis of paraffin-embedded samples. The analysis of archival material has made possible the analysis of discrete patient populations and measurement of the impact of DNA aneuploidy on relapse free and overall survival. From numerous studies reported

(over 7,000), there is growing evidence that DNA aneuploidy in solid tumors is a sign of poor prognosis. With this new prognostic information available, clinical interest in using DNA flow cytometry data to select treatment strategies for individual cancer patients has mounted. However, the data presented in Chapters 4 and 5 suggest that the technique is insufficient in certain clinical circumstances. The presence of DNA aneuploidy in primary breast tumors did not provide additional prognostic information in a subgroup of axillary node-negative patients. Furthermore, there was no correlation between DNA index and the inherent radiosensitivity of cervical and endometrial carcinomas. To conclude, DNA measurements, alone, should not have a significant impact on the design of individual treatment regimens. The complexity of factors, both genetic and environmental, that contribute to the evolution of malignancy suggest that multivariate analysis of DNA content and established prognostic factors may add valuable prognostic information to the management of individual cancer patients.

6.5 FUTURE OF DNA ANALYSIS

In the near future, flow cytometry is likely to become more useful to the clinician as more comparative studies of DNA content and prognosis will be complete. However, many of these studies have used isolated nuclei, significantly limiting the range of cellular features that can be evaluated. The development of new methods to retain extranuclear cellular components in suspensions obtained from paraffin blocks is a fertile area for future research. By maintaining the architectural integrity of the "whole cell", other variables can be examined simultaneously with DNA content to provide additional prognostic information. Examples of such multiparameter analysis include the quantitation of c-*erb*B-2 (HER-2/*neu*) oncogene protein or estrogen receptor with DNA ploidy to assess the prognosis of breast cancer patients. Moreover, with new markers to tumor

associated and differentiation antigens rapidly emerging, it may soon be possible to specifically and reliably identify tumor cells. These new markers, in conjunction with DNA measurements, may assist the clinician in the assessment of tumor status and in the identification of events associated with early neoplastic transformation.

REFERENCES

1. Hedley DW. Flow Cytometry Using Paraffin-Emedded Tissue: Five Years On Cytometry 10:229-241, 1989

2. Stephenson RA, Gay H, Fair WR, Melamed MR. Effect of Section Thickness on Quality of Flow Cytometric DNA Content Determinations in Paraffin-Embedded Tissues. Cytometry 7:41-44, 1986

3. Hedley DW, Friedlander ML, Taylor IW, Rugg C, Musgrove E. Method of Analysis of Cellular DNA Content of Paraffin-Embedded Pathological Material using Flow Cytometry. J Histochem Cytochem 31:1333-1335, 1983

4. Hiddemann W, Schumann J, Andreeff M, Barlogie B, Herman CJ, Leif RC, Mayall BH, Murphy RF, Sandberg AA. Convention on Nomenclature for DNA Cytometry. Cytometry 5:445-446, 1984

5. Harris H. The Analysis of Malignancy by Cell Fusion: The Position in 1988. Cancer Res 48:3302-3306, 1988

6. Joensuu H, Kallioniemi O-P. Different Opinions on Classification of DNA Histograms Produced from Paraffin-Embedded Tissue. Cytometry 10:711-717, 1989

7. Melamed MR, Lindmo T, Mendelsohn ML (Editors). Flow Cytometry and Sorting 2nd Edition John Wiley & Sons, Publication, New York, p. 746, 1990

CHAPTER SEVEN

CONCLUSION

Flow cytometric DNA analysis has emerged as an important prognostic parameter, particularly in patients where currently available clinicopathological criteria are incapable of predicting radically different outcomes. The inevitable growth of DNA cytometry in the clinical laboratory, both through increased use of existing applications and the introduction of new ones, is welcomed, but growth also involves increasing responsibility for those who are active in the field. It is our responsibility to ensure that this growth builds on firm foundations and that standards are set assuring excellence in practice. In addressing the subject of standardization, it was the objective of this thesis to identify the variability inherent in the procedure for processing paraffin-embedded tissue for flow cytometric DNA analysis. In doing so, several technical improvements have been suggested to develop a single optimized methodology so that findings established in one laboratory can be meaningful and reproducible in other laboratories. The second objective of this thesis was to evaluate the role of DNA flow cytometry in a clinical setting. DNA content was measured in solid tumors of breast, cervix and endometrium and found to offer limited predictive value of poor clinical outcome. This suggests that a more complex relationship exists between the generation of DNA aneuploidy and the cellular progression to malignancy. These findings stimulate a much greater enthusiasm for future multiparametric analysis of clinical samples to evaluate DNA and other cellular properties together for their prognostic potential.

APPENDIX

* Calibration Constant

  Used to generate the upper and lower channels of the tetraploid region.

  $$\frac{\text{mean channel of tetraploid population}}{\text{mean channel of diploid population}}$$

* Coefficient of Variation (CV) - a measure of resolution or precision of the flow cytometer.

  $$\%\ CV = \frac{\text{standard deviation of the peak}}{\text{mean channel of the peak}} \times 100$$

* Nuclear Concentration - determined using a hemocytometer

  $$\frac{\text{nuclei}}{\text{ml}} = \frac{\text{number of nuclei counted in 4 large outer squares}}{4} \times \frac{10^4}{\text{ml}}$$

* Quantum Yield - a measure of the efficiency of converting absorbed photons of light to emitted photons of light.

* SEM - standard error of the mean

  $$SEM = \frac{\text{standard deviation of the measurements}}{\text{number of measurements}}$$